Tumor Cell Glycolipids as Mediators of Metastasis

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Table of contents

Page

Abbreviations

CGT	Cerebroside galactosyltransferase
CR	consensus repeat domain
CST	Cerebroside sulfotransferase
ECM	Extracellular Matrix
EGF	Endothelial Grow Factor
EMT	Epithelial-mesenchymal transition
FGF	Fibroblast Grow Factor
FGFR	Fibroblast Grow Factor Receptor
Fuc	Fucose
GD1a	Neu5Acα3Galβ-3GalNAcβ-4(Neu5Acα3)Galβ-4Glcβ-1Cer
GM2	GalNAcβ-4(Neu5Acα3)Galβ-4Glcβ-1Cer
GM3	Neu5Acα3Galβ-4Glcβ-1Cer
GM4	Neu5Acα3Galβ-1Cer
HBSS	Hank's Balanced Salt Solution
HPTLC	high performance thin layer chromatography
IL1-β	Interleukin 1
LP	Lower Phase
LPS	lipopolysaccharide
MALDI-TOF-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MET	Mesenchymal-epithelial transition
MMP	Metalloproteinases
NK	Natural Killer
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PLC	phospholipase C
PPMP	D,1-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-propanol
shRNA	short hairpin RNA
siRNA	silence RNA
sLe^a	sialyl Lewis A
sLe^x	sialyl Lewis X
SM2a	GalNAcβ-4(HSO3-3)Galβ-4Glcβ-1Cer
SM3	HSO3-3Galβ-4Glcβ-1Cer
SM4	sulfatide HSO3-3Galβ-1Cer
TGF-β	Tumor Grow Factor
TNF-α	Tumor Necrosis Factor
UP	Upper Phase

Summary

Alteration of cell surface glycosylation is a common feature of cancer cells. High expression of sialylated and fucosylated glycans chains, like sLe^x and sLe^a, has been correlated with poor prognosis due to a high rate of metastasis. Selectins are vascular adhesion molecules implicated in leukocyte homing and they recognize specific carbohydrates structures. Selectins participate in cancer malignancy by binding tumor cells carrying mucins with $sLe^{x/a}$.

In this project we aimed at defining the role of sulfoglycolipids as possible selectin mediators of metastasis. P-selectin was shown to mediate metastases of mouse colon adenocarcinoma cells (MC-38) through selectin binding to ligands on these cells. Attenuation of metastases of MC-38 cells was observed in P-selectin deficient mice. The presence and nature of the P-selectin ligands on these cells was analyzed by flow cytometry. The use of enzymatic treatments of intact cells, such as desialylation and desulfation, suggested that the selectin ligand on these cells is a sulfoglycolipid. MALDI-TOF-TOF analysis of the purified sulfate-containing lipid fraction confirmed the ligand to be a sulfated galactosylceramide (SM4 sulfatide). While over-expression of sulfoglycolipids was previously reported in some cancers, its biological relevance remains unclear. We could show that enzymatic removal of sulfation from the cell surface of MC-38 cells not only resulted in reduced P-selectin binding but also led to attenuation of metastasis. A specific down-regulation of the sulfatide expression by siRNA against the responsible sulfotransferase, led to lower platelet-tumor cell aggregate formation and reduction of metastases. All these data show that sulfatides are biologically relevant ligands involved in selectin-mediated metastatic spread.

Section I

Introduction

1. Cancer

According to the American Cancer Society, cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells forming tumors. Those that grew locally without invading adjacent tissues are classified as benign. Those that invaded nearby tissues and have the capability of colonizing new tissues are called malignant.

The majority of tumors arise from epithelial tissues (**carcinomas)**; which are responsible for more than 80% of human cancer related-death in western world, the other 20% is composed by sarcomas, myelomas, leukemias, and lymphomas. The process of tumor formation involves multiple steps leading to alterations of cells and their physiological behavior. Major changes include both activation of oncogenes and inactivation of tumor suppressor genes. Tumor progression is driven by a sequence of mutations and epigenetic alterations affecting genes that control cell proliferation, adhesion, survival, and other processes associated with malignant phenotype (Hanahan and Weinberg 2000; Weinberg 2007).

In the process of tumorigenesis, epithelial cells lose their polarity leading to the exposure and secretion of aberrantly glycosylated tumor mucins to the bloodstream. Enhanced production of altered mucins is a typical process for many carcinomas and has been used as diagnostic tool for adenocarcinomas (Girling, Bartkova et al. 1989; Borsig 2004; Byrd and Bresalier 2004).

2. Tumor Biology and Metastasis

The migration of cancer cells from the original tumor site through the blood and lymph vessels to produce cancers in other tissues is called **metastasis**. This is the leading cause of death due to cancer. The establishment of metastasis is a multi-step process that requires the ability of cancer cells to invade adjacent tissues, to enter into the blood or lymphatic vessels, to migrate to other organs through these vessels and finally to leave the vessels and invade distant tissues.

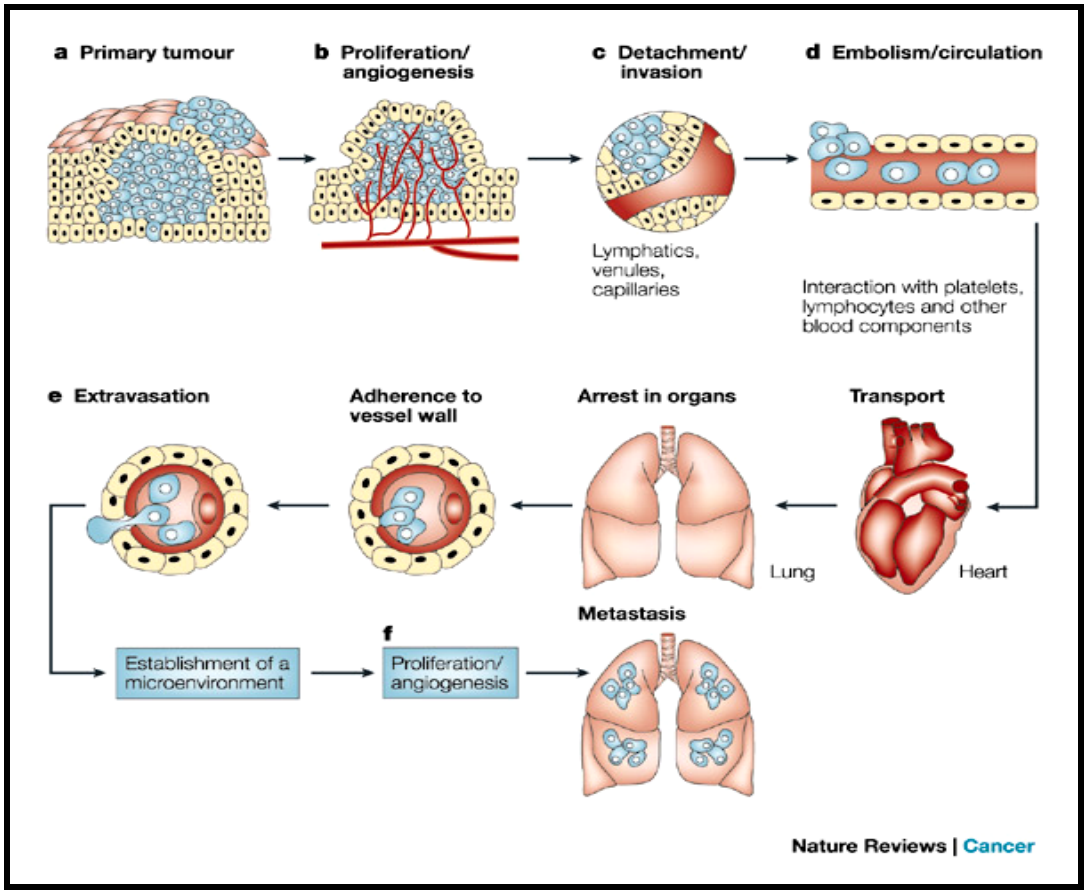

Figure 1 Steps in the metastatic process. Primary tumor cells will grow and stimulate angiogenesis. Some of the cells will acquired the ability to invade surrounding tissues until the intravasation into blood circulation where they will interact with blood components as leukocytes and platelets that will facilitate the formation of tumor cell emboli. The tumor cell emboli will arrest in a distant organ where it will extravasate and form a new metastatic colony. (Fidler 2003)

Thin sheets of epithelial cells are built atop of layers of stroma. Separating these two layers, there is a rich protein net that is constructed by proteins secreted by epithelial and stromal cells. This specialized type of extracellular matrix (ECM) is

known as the basement membrane. Carcinomas are considered benign as long as the cells forming the tumor remain on the original side of the basement membrane, while malignant carcinomas are tumors that invade the basement membrane.

In the process of malignancy an important step is **angiogenesis**; a process leading to the formation of new blood vessels. This angiogenic switch seems to be an ability that many tumor cell populations are initially lacking but must acquire during tumor progression. Angiogenic signals are induced by benign cancer cells through the basement membrane in order to increase angiogenesis on the stromal side. In the angiogenic process tumors can actively recruit endothelial cells that will construct capillaries within the tumor masses.

In order to acquire motility and invasiveness, carcinoma cells lose many of their epithelial phenotypes, detach from epithelial sheets and undergo a drastic alteration (ex. loss of cell polarity, and loss of E-cadherin resulting in release of cell–cell adhesion). It has recently been proposed that a major process contributing to tumor progression is epithelial to mesenchymal transition (EMT) (Thiery 2002) (Brabletz, Jung et al. 2005) (figure 2). EMT is also required for the reconstruction of epithelial cell layer after wounding. In the pathological process, epithelial cells stop expressing typical epithelial markers and instead express mesenchymal markers. At the same time these cells change their morphology and take on the appearance of fibroblasts.

Table 14.2 Cellular changes associated with the epithelial–mesenchymal transition

Loss of	
	Cytokeratin (intermediate filament) expression
	Epithelial adherens junction protein (E-cadherin)
	Epithelial cell polarity
Acquisition of	
	Fibroblast-like shape
	Motility
	Invasiveness
	Mesenchymal gene expression program
	Mesenchymal adherens junction protein (N-cadherin)
	Protease secretion (MMP-2, MMP-9)
	Vimentin (intermediate filament) expression
	Fibronectin secretion
	PDGF receptor expression
	αvβ6 integrin expression

Figure 2 Cellular changes associated with the epithelial-mesenchymal transition. Some of the changes that will provide the tumor cells with the ability for motility and invasiveness (Weinberg 2007)

One of the major early changes a tumor cells undergo before it becomes invasive is the loss of the transmembrane E-cadherin. This is followed by turnover of cell–substratum focal adhesions (Dennis, Granovsky et al. 1999). The mesenchymal marker N-cadherin appears after the loss of E-cadherin increasing the affinity of cancer cells for stromal cells that normally express N-cadherin. Aberrant de novo expression of N-cadherin is associated with an increased invasion potential (Hazan, Phillips et al. 2000). Increased fibroblast growth factor 2 (FGF2) signaling through N-cadherin-mediated FGF receptor 1 (FGFR1) stabilization results in an elevated matrix metalloproteinase-9 (MMP) production, which could account for the invasive phenotype induced by N-cadherin (Suyama, Shapiro et al. 2002). Another proposed mechanism is the interaction of the tumor cells with N-cadherin-positive fibroblasts of the surrounding stroma. It seems that this process is reversible and in the absence of EMT signals, the carcinoma cells may then undergo a mesenchymal-epithelial transition (MET) and revert to the original phenotype of the primary tumor. Another main change associated with malignancy of carcinoma is the alteration of cell surface glycosylation; which can also contribute to tumor invasion by altering tumor cell-cell interaction and adhesion (Dennis and Laferte 1987) (Hollingsworth and Swanson 2004).

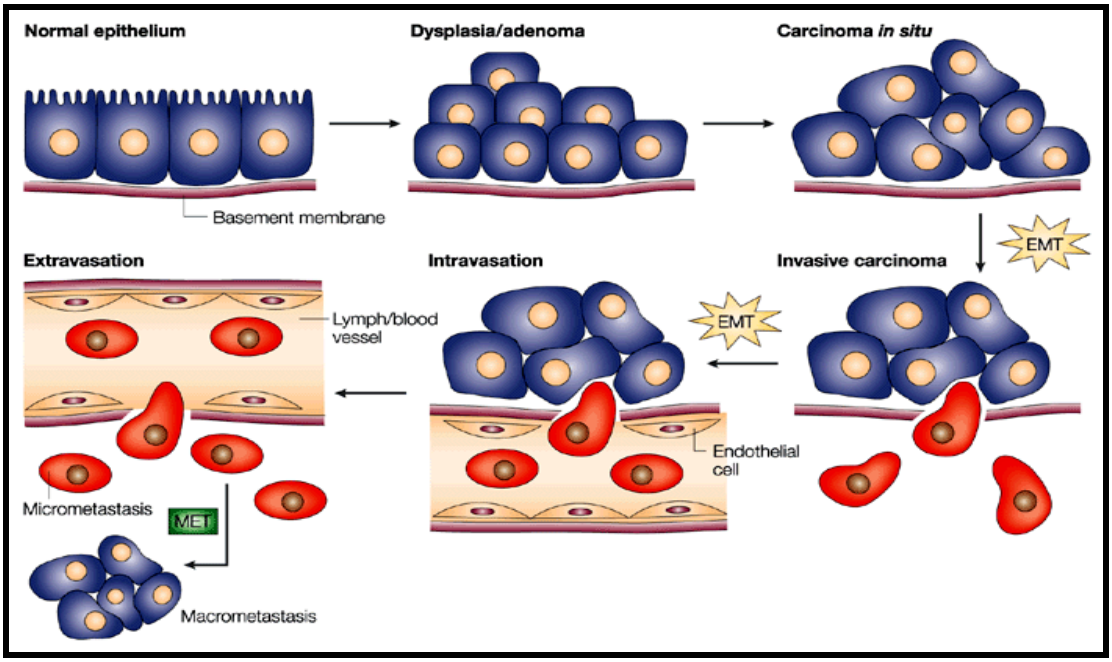

Figure 3 EMT transition. Cancer cells undergo through alterations that can induce local dissemination and invasion possibly through an epithelial—mesenchymal transition (EMT). These cells can intravasate into lymph or blood vessels and be transported to distant organs where they can extravasate form a new carcinoma through a mesenchymal—epithelial transition (MET). (Thiery 2002)

The process of invasion is facilitated by the action of different players; such as growth factors, MMPs, chemokines, among others. A typical example is the action of tumor growth factor (TGF-β) promoting the aggressiveness of malignant cancer cells (Hagedorn, Bachmeier et al. 2001). The levels of tumor TGF-β have been directly associated with the degree of tumor invasiveness and general aggressiveness in numerous studies. TGF-β can contribute to malignancy in several ways. Many human carcinomas from the intestine retain at least some functional TGF-β receptor signaling. The inactivation of retinoblastoma, tumor suppressor gene (pRb) pathway causes malignant cells to lose their response to the cytostatic effects of TGF-β. On absence of the cytostatic effects of TGF-β, the exposure of cancer cells to this factor favors proliferation.

Cancer cells also need to remodel the nearby tissue environment to become invasive by making their way through the extracellular matrix and pushing aside any cells that stand in their path. Here the most important effectors are the matrix MMP. The main components cleaved by MMPs are extracellular matrix proteins such as fibronectin, tenascin, laminin, collagens and proteoglycans. Soluble MMPs are synthesized as inactive pro-enzymes that can only function following activation by other proteases. Several cancer cells can produce MMPs but the majority of the MMPs are produced by stromal cells (macrophages, mast cells and fibroblasts).

The invasive properties tumor cells exhibit allow them to move through the wall and into the luminal site, a process called intravasation, of lymphatic or blood vessels (figure 3). The blood represents an actively hostile environment for the invading cancer cells which can be eliminated by the innate immune response (Smyth, Thia et al. 2000). Circulating tumor cells can also be torn apart by hydrodynamic shear forces in circulation (Weiss and Ward 1983). If tumor cells survive the initial steps and gain access to the larger vessels in the venous system, they will travel with the blood through the heart and then get trapped in the first set of capillaries that they encounter. Once in the blood vessels, tumor cells may encounter and interact with platelets to form the tumor cell-emboli. Leukocytes can facilitate the formation of these aggregates. The tumor cell-emboli protect cancer cells from innate immune-response. It stabilizes tumor cell arrest in the vasculature, stimulates tumor cell proliferation, promotes tumor cell extravasation by enhancing tumor cell interaction with the extracellular matrix and the endothelial cell surface (Starkey, Liggitt et al. 1984; Belloni and Tressler 1990; Honn, Tang et al. 1992). The formation of the tumor

cell-emboli contributes to the establishment of new metastases as it has been shown that various anti-platelet agents have potent inhibitory effects on tumor cell-platelet aggregation as well as on spontaneous or experimental metastases (Honn, Tang et al. 1992; Mousa 2002). Tumor cell-emboli formation at least partially has been shown to be mediated by selectins as will be discussed in the next section.

Once tumor cells are in circulation these cells must exit from the luminal side of the vessels via extravasation, and penetrate into the surrounding tissue. Tumor cell-emboli interactions with endothelial cells contribute to this process. The ability of cancer cells to extravasate may depend on many of the same properties used earlier to mediate invasion and intravasation. Cancer cells can have different strategies to extravasate by pushing aside endothelial cells, pericytes, and smooth muscle cells until they make their way through the vessel wall.

Once they are within the parenchyma of a tissue, metastasizing cancer cells may begin forming a tumor in a process called colonization. Establishment of metastatic foci is the most limiting step because the new tissue environments may not provide cancer cells with the specific needs for growth and survival factors.

The organ specificity of metastasis can depend on several factors. In 1889 Stephen Paget proposed a hypothesis called “seed and soil” postulating that the metastasizing cancer cells find a compatible home only in especially limited compatible tissues. However the seed and soil model cannot explain all metastatic patterns of human cancers. In certain cases, the preference to metastasize to a defined target organ is likely to be dictated by the layout of the vessels connecting the site of the primary tumor with the site of metastasis. This logic suggests that the frequency of metastases to an organ is governed by two parameters; (1) the frequency of tumor cell retention in a capillary bed, and (2) the capability of the tumor cell to adapt to the new microenvironment in order to colonize it. The fact that micrometastases established throughout the body stay for extended periods of time without growing into a tumor (dormant micrometastases) indicates that both mechanisms can contribute to colonization as a result of complex interactions between various types of metastasizing cells and microenvironments of the host tissues on which they land.

Selectins are vascular adhesion molecules that have been shown to play an important role in hematogenous metastasis. They are main players in our study and they are introduced in detail in the next section.

3. Selectins

The recognition of a new family of cell adhesion molecules involved in the regulation of the leukocyte trafficking started with the almost simultaneous cloning in 1989 of the three selectins; E-selectin, P-selectin and L-selectin (Varki, Cummings et al. 1999). Since then, rapid progress has been made in understanding the molecular and cellular basis of selectin-mediated interactions. Many studies have been done to identify the cellular ligands for selectins and the physiologic role played by selectins in both normal leukocyte trafficking and in a range of inflammatory and other diseases. Selectins are typical type I membrane proteins composed of a tandem array of discrete protein domains (figure 4). These domains include a C-type lectin domain, a single epidermal growth factor (EGF)-like domain, two to nine short consensus repeat (CR) domain, a single transmembrane region and a cytoplasmic tail.

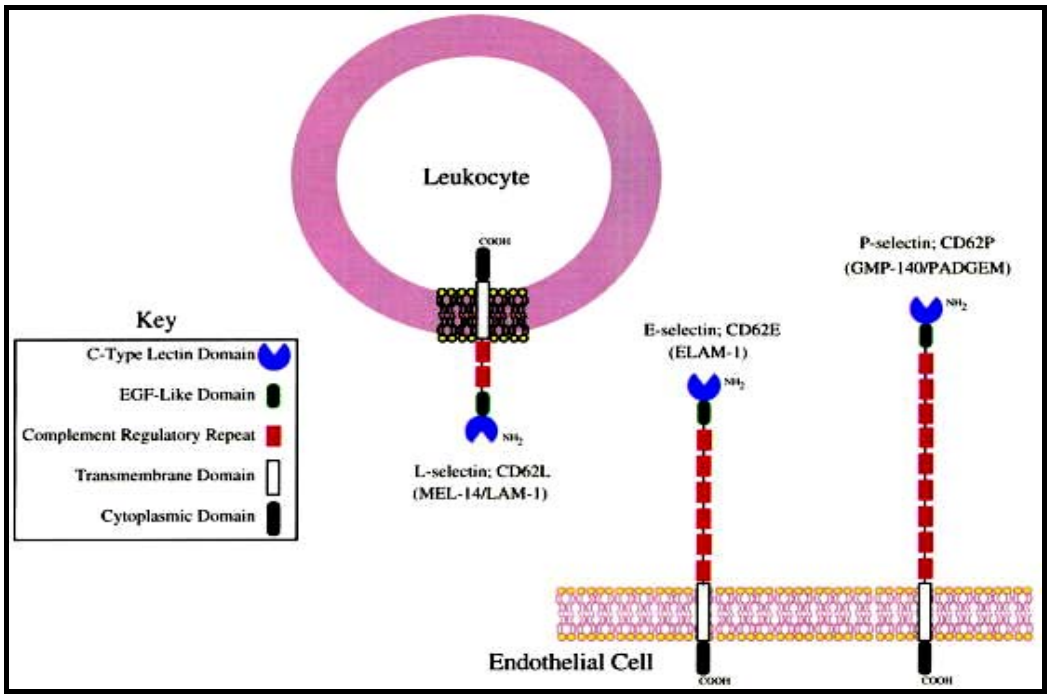

Figure 4. **Representation of the E, P and L-selectin.** L-selectin is expressed on leukocytes, E-selectin is expressed on activated endothelial cells and P-selectin is expressed on activated platelets and activated endothelial cells (Varki, Cummings et al. 1999)

Selectins share an N-terminal carbohydrate-recognition domain that is homologous to other Ca^{2+} dependent mammalian lectins (Varki 1994). They share an overall structure and primary sequences and exhibit similarities in the associated glycans that contribute to their adhesion function, thrombosis, and inflammation. All

three selectins are first response genes in cell adhesion and they have been shown to mediate the initial step of rolling of leukocytes (figure 5) (Kansas 1996).

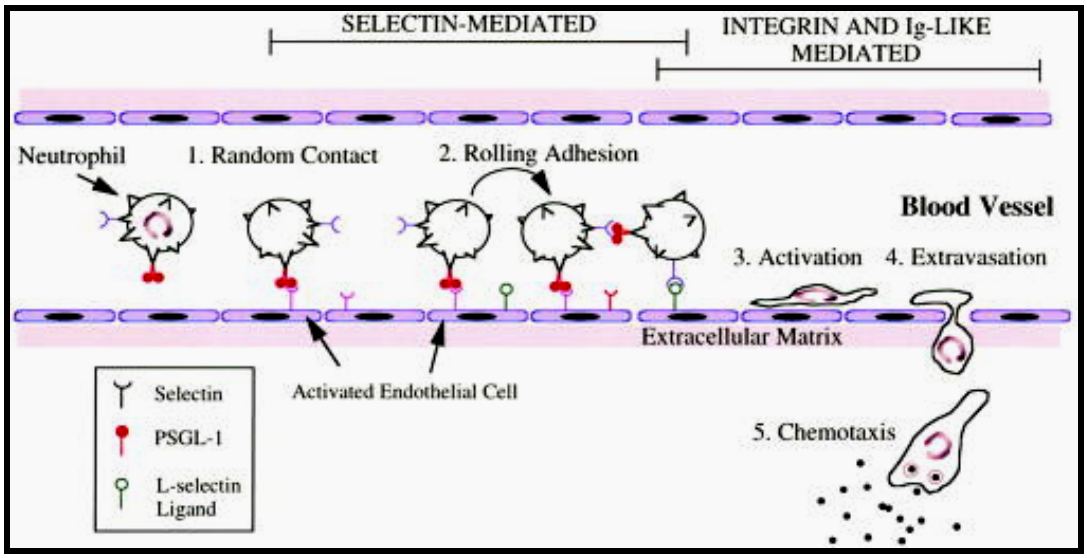

Figure 5. **Leukocyte homing mediated by selectins.** Representation of the rolling adhesion of circulating leukocytes to activated endothelium via selectins interactions with their ligands (Varki, Cummings et al. 1999)

Selectin ligands:

Selectins recognize specific carbohydrate ligands. Most of these ligands carry sialylated, fucosylated, and/or sulfated sequences normally found at the non-reducing termini of N-linked or O-linked oligosaccharides or on glycosphingolipids (figure 6). Since sugar chains are more flexible than polypeptides, they may also be more capable of accommodating structural changes while still preserving critical three-dimensional features for recognition. Selectins can recognize a discontinuous carbohydrate epitope composed of carbohydrates attached to distinct carbohydrate chains or amino acids of a glycoprotein. Despite the fact that there are many ligands recognized *in vitro* not all seem to have biological relevance (figure 7). The common feature of most of the ligands recognized by all selectins is the *N*-acetyllactosamine backbone of the type II (Galβ1,4Glc-NAc) rather than type I (Galβ1,3GlcNAc), with sialic acid (NeuAc) residue in α2,3 linkage and fucose (Fuc) residue in either α1,3 or α1,4 linkage. Selectin-dependent cell adhesion requires both sialic acid and fucose residues in a specific position and linkage, like sialyl Lewis x and sialyl Lewis a (figure 6).

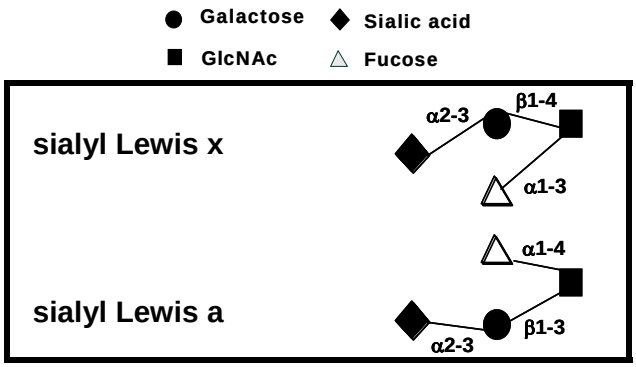

Figure 6. **Main selectin ligands.** Structure of the sLe^x and sLe^a epitopes which are recognized by selectins

The importance of the Le^x and Le^a structures makes fucosyltransferases as crucial regulatory enzymes in selectin-ligand biosynthesis. Selectins can also recognize glycosaminoglycan chains in a Ca^{2+} dependent manner. Glycosaminoglycans are linear polymers of repeating disaccharide units that do not contain sialic acid or fucose but have a variable number of sulfate esters and uronic acids. Other ligands for selectins are molecules such as sulfatides which bind to some selectins, but partially in a Ca^{2+} independent manner. These sulfatides are ligands of special interest for our project and they will be discussed in more detail below.

Table 1. General classes of molecules reported to be recognized by selectins

Class of molecules	Sources	Recognized by		
		E-selectin	P-selectin	L-selectin
Sialylated fucosylated lactosamine oligosaccharides	Widespread on N- and O-linked oligosaccharides, and on glycolipids	++	++	+
Sulfated fucosylated lactosamine oligosaccharides	Tumor mucins, some normal mucins	+	++	++
3-Sulfated glucuronosyl lipids	Brain glycolipids; some gps?	−	+	++
Sulfatide	Various sources	−	++	+
Heparin (commercial)	Pig gut or bovine lung	−	++/−	++/−
Heparan sulfate proteoglycans	Cultured calf and human endothelial cells	−	++++	++++
Chondroitin sulfate	Various sources	−	+/−	+/−
Polyphosphomannan	Phosphorylated yeast polysaccharide	−	−	++
Fucoidan	Sulfated fucosylated seaweed polysaccharide	−	++	++
Dextran sulfate	Semi-synthetic sulfated polysaccharide	−	++	++
Sialylated fucosylated mucin-type gps	Human and murine neutrophils, HL-60 cells	++	++++	
Sialylated, sulfated, fucosylated mucin-type gps	Mouse lymph nodes	++		++++
Sialoglycoproteins, type unspecified	Human, bovine, and murine leukocytes	++++	++++	

Recognition (based on binding or inhibitory capacity) by the selectins is indicated on an arbitrary scale of ++

Figure 7. **General classes of molecules recognized by selectins.** List of molecules known to be recognized by selectins (Varki 1994)

Leukocytes express one main glycoprotein (PSGL1); which is recognized with high affinity by all three selectins. Despite its low availability, PSGL1 is the main selectin ligand expressed on leukocytes. The sialic acid is a critical determinant required for P-selectin recognition of this protein since sialidase treatment completely abolishes the binding of P-selectin to neutrophils. In addition other studies showed the importance of sulfation since the removal of sulfate from tyrosine in PSGL-1 abrogates the binding of the protein to P-selectin. It has been observed that PSGL-1 expressed by myeloid cells acts as a disulfide-bonded protein which enhances the avidity for P-selectin recognition. PSGL-1 binding to P-selectin is characterized by high-affinity binding and high association-dissociation rates.

L-Selectin:

L-selectin is expressed on all blood neutrophils and monocytes, and on the majority of blood borne T and B cells, on a subset of natural killer (NK) cells, and on immature hematopoetic cells. The cytosolic domain of L-selectin is also involved in signal transduction processes, since cross-linking of L-selectin can lead to intracellular phosphorylation and subsequent signal transduction. L-selectin is rapidly lost from the surface of normal leukocytes in response to a variety of stimuli. This shedding involves proteolytic cleavage immediately adjacent to the outer surface of the cell membrane. The very first steps of leukocytes rolling are mediated by L-selectin adhesion (figure 5) as it was shown that L-selectin deficient mice have an impaired homing of naive L-selectin-positive T lymphocytes to peripheral and mesenteric lymph nodes. This deficit is associated with a decreased immune response. The adhesive interactions of selectins operate predominantly under conditions of vascular shear flow.

In addition to its role in normal lymphocyte recirculation, L-selectin directly mediates rolling of leukocytes on endothelium at sites of inflammation or tissue injury. L-selectin acts both in early and in late inflammatory response. L-selectin deficient mice have some defect in neutrophil extravasation in sites of inflammation confirming the role for neutrophil L-selectin in adhesion to vascular endothelium.

E-Selectin:

E-selectin expression is limited the endothelium. It is expressed on endothelial cells after inducing transcription of the E-selectin gene. TNF-α, IL1-β and lipopolysaccharide (LPS) mediated the inducible transcription of the E-selectin locus. Cytokine dependent regulation of E-selectin locus increases E-selectin expression approximately 2 hours after cytokine treatment, with maximum expression at 4 hours and it will go back to basal levels within 10 to 12 hours.

E-selectin supports rolling of leukocytes at sites of inflammation and tissue injury. In the mouse, P and E-selectin are coexpressed in many inflamed tissues. E-selectin appears to play an important role as a tissue specific homing receptor for leukocyte recruitment; specifically to the skin. E-selectin expression is associated with infiltration of T-cells in many dermatological disorders. It seems that P and E-selectin maintain a certain functional redundancy although the time scale of their action is different. In E-selectin deficient mice no defect on leukocyte recruitment in sites of inflammation was observed unless P-selectin function was simultaneously blocked with antibody.

E-selectin ligands, like PSGL1, are mainly expressed by neutrophils, monocytes, eosinophils, memory-effector T-like lymphocytes, and natural killer cells. These are cells found primarily in acute and chronic inflammation sites.

P-Selectin:

P-selectin is found stored within the Weibel-Palade bodies of endothelial cells and in α-granules of platelets, playing a dual role in blood coagulation hemostasis and inflammation. P-selectin contributes to leukocyte recruitment in a variety of acute and chronic inflammation. Leukocyte recruitment and inflammation is a result of both acute and chronic expression of P-selectin by the endothelium and by platelet activation-dependent P-selectin expression as one of the first responses in this context. P-selectin deficient mice exhibit a substantial delay in neutrophil recruitment during acute inflammation, indicating that P-selectin expression is a first response after activation playing an active role in the early phases of leukocyte recruitment.

P-selectin can be expressed within minutes after activation. Cell surface expression of P-selectin is generally transient and is also under transcriptional control of factors including TNF-α, IL1-β or LPS, with a time course similar to the one of E-selectin.

Activated platelets expressing P-selectin contribute to leukocyte trafficking, as well as to wound healing and blood clotting (Subramaniam, Frenette et al. 1996). They contribute to hemostatic processes via their ability to stimulate monocytes tissue factor expression that leads to leukocyte accumulation in areas of vascular injury associated with thrombosis and inflammation (Furie, Furie et al. 2001). Studies in selectin deficient mice showed that P-selectin plays a predominant role in leukocyte recruitment as one of the first responses mediating cell-cell interaction. P-selectin is longer than E and L-selectin, allowing it to extend more easily over the negatively charged glycocalix and therefore of forming a more efficient interactions between leukocytes and endothelial cells (Robinson, Frenette et al. 1999).

P-selectin binding is inhibited *in vitro* by many different anionic compounds. These includes sulfated glycoconjugates, (heparin, sulfatides), and phosphorylated compounds (figure 7). However, only a few of them have been shown to have a biological relevance as P-selectin ligand *in vivo*.

Selectins & Cancer:

Besides their role in homeostasis and inflammation, selectins have been shown to be active players in cancer because of their role in the formation of the tumor cell-emboli.

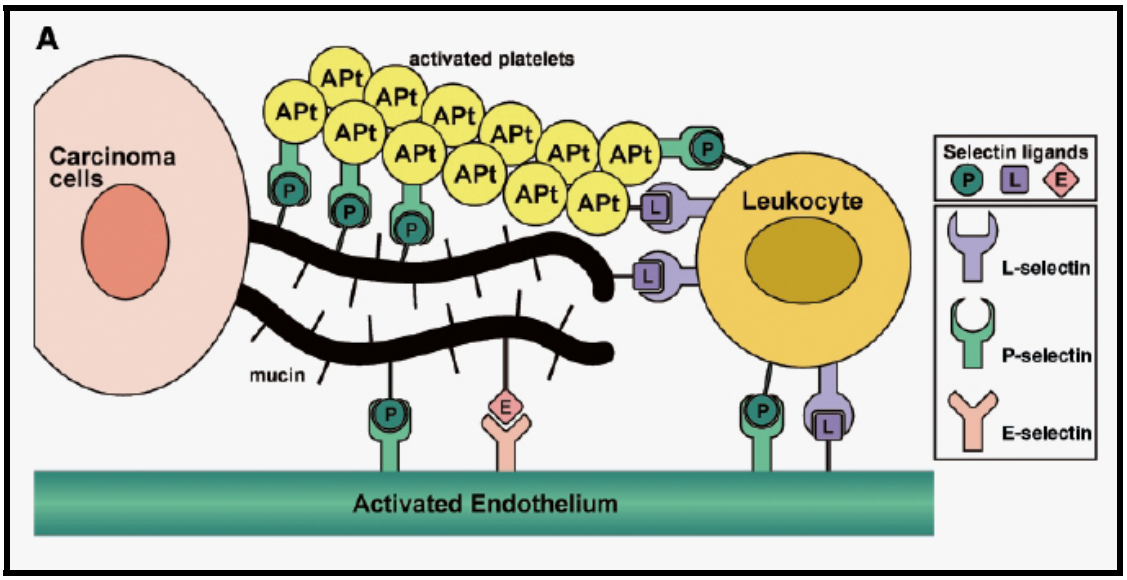

Figure 8. **Selectins mediation of metastasis.** Hypothetical model showing all the interactions that can take place between carcinoma cells carrying selectin ligands and selectins expressed on activated platelets, activated endothelial cells and leukocytes. All of these interactions have been shown to occur in vitro and some have been shown in vivo. (Borsig 2004)

Malignant carcinoma cells often express carbohydrate determinants like sialyl Lewis A and sialyl Lewis X. These structures can serve as ligands for the selectin family of molecules (Kannagi 1997; Izawa, Kumamoto et al. 2000). They are involved in the adhesion of cancer cells and can contribute to hematogenous metastasis of cancer via the enhancement of cell to cell interaction mediated by selectins. The exposure of these determinants is usually associated with a poor prognosis due to a high rate of metastasis (Nakamori, Kameyama et al. 1993). Once tumor cells expressing selectin ligands are in the vasculature they could interact with selectins to form the tumor cell-emboli through interactions with platelets P-selectin, leukocytes L-selectin and or endothelial E & P-selectin (figure 8). These interactions could protect the tumor cell from the native immune system, facilitating the arrest and extravasation. This role of selectins in cancers has been shown in several works (Orr, Wang et al. 2000; Borsig, Wong et al. 2002). Artificially over-expressed E-selectin in the liver of transgenic mice resulted in a redirection of the metastases in liver of tumor cells that normally colonize lung as a consequence of this over-expression (Biancone, Araki et al. 1996). It has been shown that P-selectin deficiency attenuates tumor growth and metastasis (Kim, Borsig et al. 1998). These interactions between tumor cell, leukocytes, platelets and endothelial cells mediated through selectins play a critical role for metastases. The tumor cell emboli protect the tumor cells from the host defense and promote retention into capillaries which could lead to colonization of more distant organs. P- and L-selectin double null mice had a lower metastatic rate than the single selectin deficient mice, showing the synergy of selectins in cell to cell interactions. Heparin is known to be an inhibitor of selectins. The reduction in metastases of P- & L-selectin deficient mice could be reproduced by injection with heparin (Borsig, Wong et al. 2001), (Borsig, Wong et al. 2002).

4. Sulfatides

A class of sulfoglycolipids, containing sulfate esters on carbohydrates, is called sulfatides. They contain a hydrophobic ceramide moiety and a hydrophilic moiety which consists of mono- or oligosaccharide unit bound to the C-1 hydroxyl group of the ceramide. Generally, the terminal galactose residue is esterified with sulfate at carbon 3. The original use of this term was to designate sulfo-galactosyl ceramide, SO_3-Galβ-1Cer (SM4).

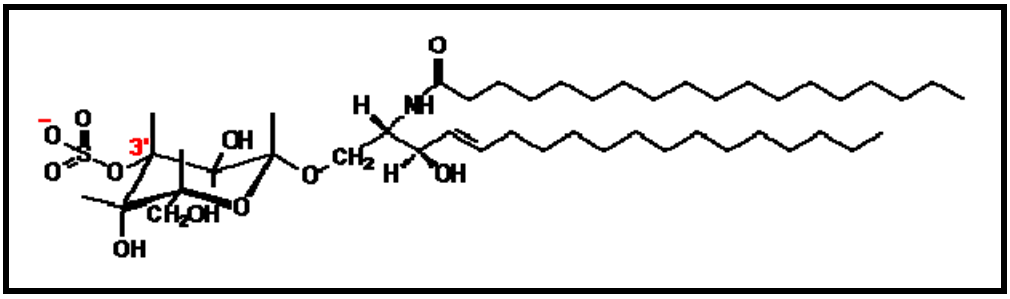

Figure 9. Structure of sulfo-galactosyl ceramide, SO_3-Galβ-1Cer (SM4)

SM4 represents the first sulfatide isolated in 1884 from human brain. SM4 is a component of the plasma membrane and its presence is enriched in glandular epithelial tissues of mammals and increased in organs related to sodium excretion. Mammalian epithelial tissues contain a variety of sulfatides and they are expressed on the outer leaflet of the plasma membrane of the glandular epithelial cells (Sugano, Tai et al. 1995). SM4 is a major sulfoglycolipid of the mammalian nervous system, kidney, and spleen (Tadano-Aritomi and Ishizuka 2003). It is also expressed in keratinocytes, lung, central nervous system, peripheral nervous system, erythrocytes, platelets, granulocytes, and in the gastrointestinal tract. In the nervous system, sulfatide is an integral constituent of myelin membranes of the central nervous system and it is biosynthesized by oligodendrocytes, the cells producing myelin, but not in neurons and astroglia (Ishizuka 1997). The carbohydrate structures of the major animal sulfatides, SM4, SM3, SM2a and Sb1a are similar to the corresponding sialic acid analogues, gangliosides GM4, GM3, GM2 and GD1a, respectively (figure 10).

Sulfatides have been shown to interact specifically with cell adhesive proteins including thrombospondins, laminins, and vascular proteins including selectins, as

well as with pathogenic viruses and mycoplasmas (Kobayashi, Honke et al. 1994; Ding, Kawashima et al. 2000; Zhong Wu, Honke et al. 2004).

***Biosynthesis of sulfatides*:**

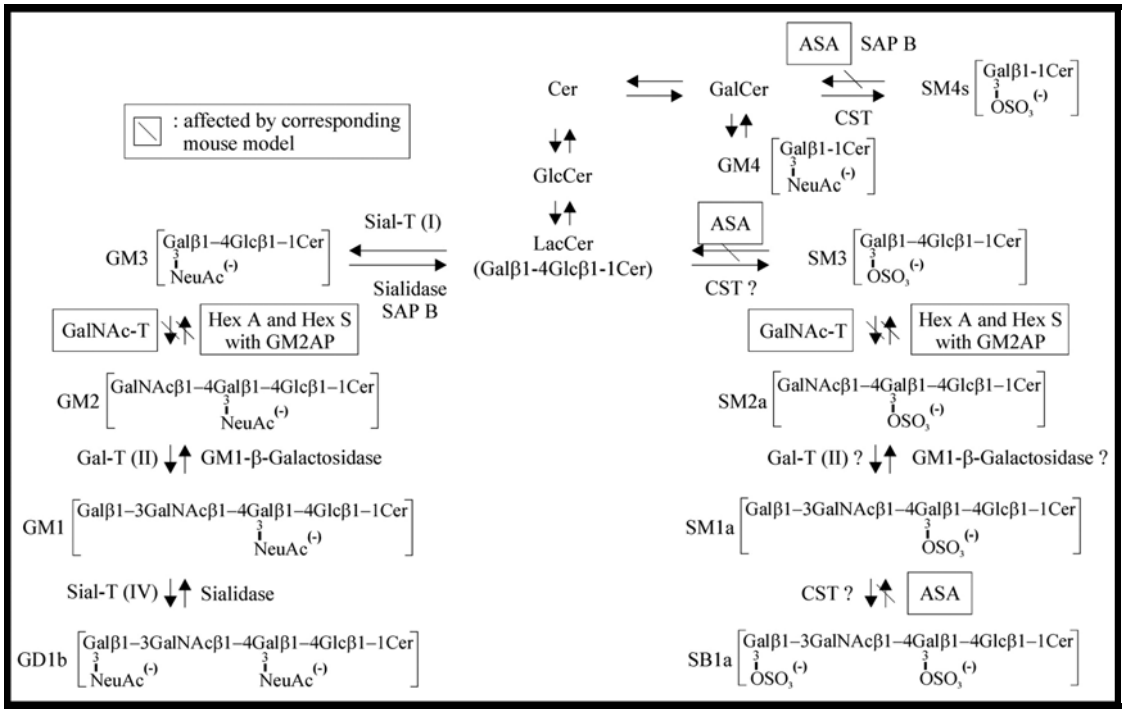

Figure 10. **The synthetic pathways of sulfolipids** (Sandhoff, Hepbildikler et al. 2002)

Ceramide sulfotransferase (CST) is the only enzyme responsible for the synthesis of sulfatides by the transfer of the sulfate group at position 3 of the galactose. The expression of CST gene is tissue specific (Hirahara, Tsuda et al. 2000). CST activity was demonstrated in the Golgi-rich fraction of rat kidneys, rat testis and Schwann cell line. This localization in the lumen of the Golgi apparatus is similar to that of glycosaminoglycan sulfotransferases and tyrosine sulfotransferases. The site of sulfation may be localized in the distal Golgi or trans-Golgi network because brefeldin, which destroys distal to medial Golgi, inhibits SM4 synthesis completely. However Gal-Ceramide remained intact, indicating that the ceramide galactosyltransferase (CGT) is located in the endoplasmic reticulum.

The best substrate for CST is GalCer while LacCer is less efficient. The way galactose is presented by the glycolipids is essential for this enzyme to recognize the acceptor and transfer the sulfate. It was shown that galactose alone did not serve as a

substrate indicating the need of the lipid moiety for recognition. The lack of sulfatide in CST deficient mice resulted in neurological disorders due to myelin dysfunction and an arrest of spermatogenesis (Honke, Zhang et al. 2004).

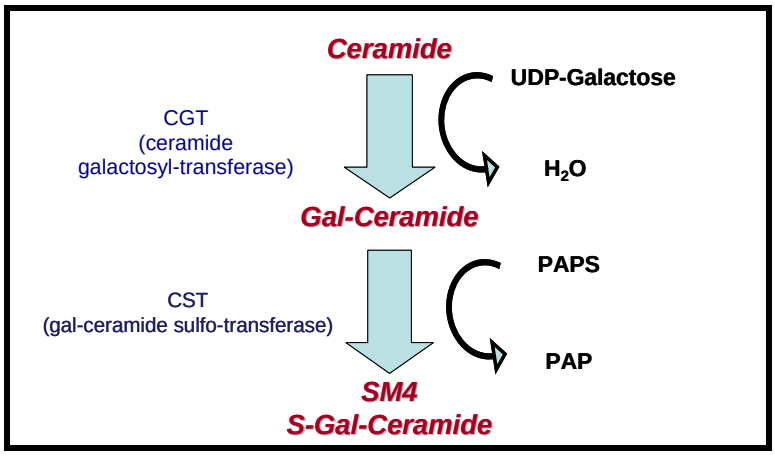

Figure 11. **Main enzymatic pathway implicated in the synthesis of SM4.** CST uses PAPS as sulfate donor for the synthesis of sulfatides

Sulfatides & selectins:

P-selectin can bind efficiently SM4 on the cell surface of granulocytes and several myeloid cells (Bajorath, Hollenbaugh et al. 1994). The sulfatide interactions with P-selectin stabilize platelet aggregates, representing a new mechanism of platelet aggregation that may play a significant role in hemostasis and thrombosis (Merten and Thiagarajan 2001). It has been shown that SM4 specifically triggered the increase of cytosolic free Ca^{2+} in neutrophils. This is likely through the interaction with L-selectin. Additionally the secretion of TNF-α and IL-8 in monocytes and neutrophils by SM4 may correspond to a similar reaction elicited by LPS. Sulfatide has been shown to be biological relevant L-selectin ligand in the kidney and it plays a central role in monocyte infiltration into the kidney interstitium (Ogawa, Shikata et al. 2004).

Sulfatides & cancer:

Sulfatide expression has been observed in several carcinomas. High expression of CST correlates with the accumulation of sulfatides in several cases of gastric cancer (Kobayashi, Honke et al. 1994; Kobayashi, Honke et al. 1999). Other studies showed that sulfatides on renal-cancer cells are involved in attachment to

laminin and that they can modulate the metastatic potential of renal-cell carcinoma cells (Kobayashi, Honke et al. 1994). SM4 content was found 10 fold higher in pulmonary adenocarcinoma when compared with the normal lung which was accompanied by an elevated CST activity. In addition, sulfatides enhance the aggregation of platelet-leukocyte aggregates. These mechanisms may play a significant role in hemostasis and thrombosis (Merten, Beythien et al. 2005). There are data supporting that changes in sulfatide composition may play an important role in lymph node metastasis of colorectal adenocarcinoma (Morichika, Hamanaka et al. 1996). When SM3 expression was induced in hepatocellular carcinoma (HCC) enhanced metastasis was observed. Thus, SM3 was associated with interaction with extracellular proteins (Zhong Wu, Honke et al. 2004).

5. Aim of the project

The aim of this project was to investigate sulfatide expression on cancer cells as potential ligands for selectin mediated metastasis. Selectins are implicated in the process of metastasis mediating a variety of interactions involving tumor cells, platelets, leukocytes and endothelial cells. Three specific objectives were pursued in this work: 1) the characterization and identification of sulfoglycolipids on tumor cells. 2) the alteration of sulfatide expression and analysis of selectin binding. 3) the study of the effect of sulfatide expression on metastatic progression.

Although many selectin ligands have already been described, this work provides a direct evidence for sulfoglycolipids on cancer cells as biologically functional ligands in selectin mediated metastasis.

Section II

Results

10/4/06

Glycobiology

P-selectin mediates metastatic progression through binding to sulfatides on tumor cells

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Running Title: P-selectin binds tumor cell sulfatides

Key words: carbohydrate sulfation / glycolipids / MALDI-TOF / metastasis / selectin

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Abstract

Hematogenous carcinoma metastasis is associated with tumor cell emboli formation, which is now known to be facilitated by selectins. P-selectin-mediated interactions of platelets with cancer cells are based mostly on mucin- and glycosaminoglycan-type selectin ligands. We previously showed that mouse colon carcinoma cells (MC-38) carry P-selectin ligands of non-mucin origin, which were not identified. Here we show that P-selectin ligands recognized on MC-38 cells are sulfated glycolipids, thereby facilitating experimental metastasis in a syngeneic mouse model. Metabolic inhibition of sulfation by incubation of cells with sodium chlorate almost completely abrogated P-selectin binding. Metabolic labeling of MC-38 cells with ^{35}S sulfate revealed only a single band as detected by HPTLC analysis of a total lipid extract. MALDI-TOF-TOF analysis of the purified sulfate-containing lipid fraction identified the selectin ligand to be a sulfated galactosylceramide (SM4 sulfatide). Modulation of glycolipid biosynthesis in MC-38 cells altered P-selectin binding, thereby confirming sulfoglycolipids to be major P-selectin ligands. In addition, P-selectin was also found to recognize lactosylceramide sulfate (SM3) and gangliotriaosylceramide sulfate (SM2) in human hepatoma cells. Finally, the enzymatic removal of sulfation from the cell surface of MC-38 cells resulted in decreased P-selectin binding and led to attenuation of metastasis. Thus, SM4 sulfatide serves as a native ligand for P-selectin contributing to cell-cell interactions and to facilitation of metastasis.

Introduction

A common phenotypic change associated with malignancy is the dramatic alteration of cellular glycosylation. Differences in expression of glycoproteins and glycosphingolipids were predominantly defined by monoclonal antibodies (Hakomori 1996; Kim and Varki 1997; Kannagi, Izawa et al. 2004). Structural identification of glycolipid antigens revealed that not all glycans are uniquely "tumor-specific", yet are predominantly present on tumors. Significant correlations between aberrant glycosylation of primary tumors and clinical prognosis have stimulated interest into identification of their biological function (Irimura, Nakamori et al. 1993; Kim and Varki 1997; Kannagi, Izawa et al. 2004). Tumor cell carbohydrates were shown to participate in cell-cell interactions, and glycosphingolipids in particular were identified as potential effectors of signal transduction (Hakomori 1985; Kannagi, Izawa et al. 2004).

Sulfatides are a class of sulfate-containing glycosylceramides distributed in various tissues, including the brain, kidney and gastrointestinal tract (Natomi, Saitoh et al. 1993; Ishizuka 1997). Biosynthesis of sulfatides requires transfer of sulfate to the glycolipid moiety, which is catalyzed by a unique cerebroside sulfotransferase (CST) (Honke, Tsuda et al. 1997; Hirahara, Tsuda et al. 2000). The physiological function of sulfated glycosylceramides has been investigated in mice lacking the CST gene, which caused a complete absence of sulfatides (Honke, Hirahara et al. 2002; Ishibashi, Dupree et al. 2002). The lack of sulfatides resulted in neurologic disorders and arrest of spermatogenesis. Several studies provided evidence that sulfatides expressed on cell surfaces of different cells exert biological functions through mediating interactions with various proteins, such as laminin, thrombospondin, amphoterin, selectins, galectin and hepatocyte growth factor (Roberts, Rao et al. 1985; Aruffo, Kolanus et al. 1991; Suzuki, Toda et al. 1993; Kobayashi, Honke et al. 1994; Shikata, Suzuki et al. 1999; Rouhiainen, Imai et al. 2000; Merten and Thiagarajan 2001; Ideo, Seko et al. 2005). Some of these proteins are adhesion molecules that are involved in cell-cell and cell-extracellular matrix interactions. In this context, selectins and laminin were shown to be involved in metastasis (Roberts, Rao et al. 1985; Aruffo, Kolanus et al. 1991; Kobayashi, Honke et al. 1994; Shikata, Suzuki et al. 1999).

Selectins are vascular adhesion molecules involved in leukocyte trafficking, inflammation, thrombosis, autoimmunity and cancer (Varki 1994; Kansas 1996). The selectin family (P-, E-, and L-selectin) recognizes sialyl Lewis$^{x/a}$-containing structures, which are normally found on mucin-type glycoproteins of leukocytes and endothelium (Dennis and Laferte 1987; Nakamori, Kameyama et al. 1993; Hakomori 1996; Kannagi 1997). The observed correlation between sialyl Lewis$^{x/a}$ expression and a poor prognosis due to tumor progression and metastasis (Nakamori, Kameyama et al. 1993; Hakomori 1996) was further validated in different mouse models, where E-, P- and recently also L-selectin were associated with metastatic progression (Biancone, Araki et al. 1996; Kim, Borsig et al. 1998; Borsig, Wong et al. 2001; Borsig, Wong et al. 2002; Ludwig, Boehme et al. 2004). In addition to sialylated and fucosylated mucin structures, P and L-selectin are able to efficiently bind sulfatides (Aruffo, Kolanus et al. 1991; Suzuki, Toda et al. 1993; Bajorath, Hollenbaugh et al. 1994; Ishizuka 1997). L-selectin mediation of monocyte infiltration in renal interstitial inflammation was shown to be facilitated by sulfatides, thus demonstrating sulfatides to be endogenous ligands of L-selectin (Shikata, Suzuki et al. 1999). Human neutrophils could be activated by sulfatides through L-selectin binding (Laudanna, Constantin et al. 1994). Additionally, P-selectin was shown to bind efficiently sulfatides on the plasma membranes of granulocytes and human tumor cells *in vitro* (Aruffo, Kolanus et al. 1991).

Elevated levels of sulfatides were detected in gastric, hepatocellular and renal cell carcinomas (Hiraiwa, Fukuda et al. 1990; Kobayashi, Honke et al. 1994; Morichika, Hamanaka et al. 1996), which was shown to be due to upregulation of CST (Morichika, Hamanaka et al. 1996; Ishizuka 1997; Honke, Tsuda et al. 1998; Zhong Wu, Honke et al. 2004). Significantly higher expression of sulfatides in colorectal carcinomas and ovarian carcinomas was correlated with poor prognosis (Morichika, Hamanaka et al. 1996; Makhlouf, Fathalla et al. 2004). Sulfatides were shown to mediate carcinoma adhesion to laminin and vitronectin, thereby modulating metastatic potential of renal cell and hepatocellular carcinomas (Kobayashi, Honke et al. 1994; Zhong Wu, Honke et al. 2004). Alteration of glycolipid expression, including expression of sulfatides, led to specific reduction of laminin-mediated attachment and migration (Inokuchi, Jimbo et al. 1990). Although sulfatide-mediated

interactions of carcinomas with selectins were shown *in vitro*, their potential to facilitate metastasis had not been investigated.

Carcinoma-associated P-selectin ligands are mostly sialylated fucosylated glycans on tumor cell mucins (for review see ref. (Byrd and Bresalier 2004). Previously, we suggested the possibility that sulfoglycosylceramides could be involved in selectin-mediated metastasis (Borsig, Wong et al. 2002). Here we validate sulfatides as functional P-selectin ligands and evaluate their potential to facilitate metastasis of mouse carcinoma cells.

Results

Mouse colon carcinoma cell line MC-38 contains sulfated selectin ligands

Previously, we have shown that MC-38 cells possess ligands for L- and P-selectin (Borsig, Wong et al. 2002). However, the nature of the ligands had not been fully determined. To identify the functional selectin ligands, MC-38 cells were stained with recombinant selectin chimeras after treatment with different carbohydrate-degrading enzymes and analyzed by flow cytometry. Initial analysis has shown that P-selectin ligands on MC-38 cells are not mucins (Borsig, Wong et al. 2002). Therefore, we checked for other potential selectin ligands, glycosaminoglycans and sulfatides (Varki 1997). Treatment of carcinoma cells with a mixture of glycosaminoglycan-hydrolyzing enzymes (heparinase and chondroitinase) did not affect binding of P-selectin, but partially decreased recognition by L-selectin (Figure 1A). The removal of sulfate groups by arylsulfatase treatment resulted in a clear reduction of P-selectin binding, as well as a reduction of L-selecting binding, although to a lesser extent (Figure 1A). While L-selectin binding to MC-38 cells was calcium-dependent, the binding of P-selectin was only partially affected by the absence of calcium (Figure 1A and (Borsig, Wong et al. 2002). These data suggested that L-selectin ligands on MC-38 cells are represented partially by glycosaminoglycans, which was in agreement with the EDTA sensitivity (Koenig, Norgard-Sumnicht et al. 1998). Earlier we have shown that L-selectin binds also mucin-type ligands (Borsig, Wong et al. 2002). Meanwhile, P-selectin recognized a unique type of ligands in a calcium independent manner. Together with the requirement for sulfation, but independent of glycosaminoglycans, the nature of

the P-selectin ligand appeared to be sulfoglycolipids (Needham and Schnaar 1993; Koenig, Norgard-Sumnicht et al. 1998).

To confirm that MC-38 cells interact also with P-selectin-expressing cells, we tested the adherence of these cells to activated platelets. Slides with immobilized activated platelets were incubated with MC-38 cells while shaking on an orbital shaker (Figure 1B). Tumor cells adhered readily to activated platelets, and this adherence was blocked by function-blocking anti-P-selectin antibody. Arylsulfatase treatment also led to reduction of adherence, albeit to a lesser extent. The incomplete inhibition of tumor cell-platelet interactions by removal of sulfation corresponded to incomplete reduction of P-selectin binding to MC-38 cell (Figure 1A). Finally, there was no adherence of MC-38 cells to non-activated platelets (data not shown). Thus cell-surface ligands of MC-38 cells enable the adherence to platelets in a P-selectin-dependent manner.

P-selectin binds to ligands in lipid fractions of MC-38 cells

To check whether P-selectin recognizes sulfated glycolipids as functional ligands on MC-38 cell surfaces, total lipid extract from these cells was prepared. Isolated lipids were separated by Folch partitioning into a lower phase (LP lipids), consisting of neutral glycolipids smaller than tetrasaccharides, together with phospholipids and sulfated glycolipids (sulfatides), and an upper phase (UP lipids) containing mostly gangliosides. Lipid fractions were coated on ELISA plates and analyzed by P-selectin chimera binding (Figure 2A). Sulfated galactosylceramide (SM4), a recognized P-selectin ligand, was used as a control (Aruffo, Kolanus et al. 1991). While P-selectin bound to LP lipids, there was no recognition of UP lipids (data not shown). The extent of P-selectin binding to LP lipids was comparable to the recognition of SM4. The LP lipids from Folch partition contain sulfatides and phospholipids, which are both known potential P-selectin ligands (Malhotra, Taylor et al. 1996). To confirm that sulfation is a prerequisite for P-selectin recognition, LP lipids were treated with arylsulfatase prior to P-selectin chimera binding. Desulfation of the LP lipids reduced binding of P-selectin by more than 55% as compared to about a 45% reduction of binding to SM4 (Figure 2A). P-selectin binding to LP lipids was only partially affected by the presence of EDTA, which was similar to P-selectin recognition of SM4. Recognition of the LP lipids was not affected by

neuraminidase treatment confirming that the P-selectin binding is independent of sialic acid (Borsig, Wong et al. 2002).

To identify P-selectin ligands in LP and UP lipid fractions, lipid samples were separated by HPTLC (Figure 2B-C). The large amount of phospholipids in the LP lipid fraction was hydrolyzed by treatment with phospholipase C prior to separation on TLC plates. Visualization of glycolipids by orcinol-sulfuric staining revealed the presence of a broad panel of glycolipids. The majority of glycolipids in the LP phase (lane 2) were migrating slower than the sulfatide SM4 (lane 1). Developing conditions for HPTLC were chosen so that the best resolution of short oligosaccharides and sulfatides would be achieved. Thus, an accumulation of gangliosides with minimal migration was visible in the UP lipid fraction (lane 3). Despite the large variety of lipids in each fraction, P-selectin bound only to two distinct lipid bands in the LP fraction of MC-38 cells (Figure 2C). There was no binding to lipids in the UP fraction, suggesting no recognition of sialic acid containing gangliosides (Borsig, Wong et al. 2002). As a positive control, SM4 was used, while a neutral glycolipid marker served as a negative control (lane 1, 4). The lipids recognized by P-selectin corresponded to a band with high mobility that migrated close to the SM4 sulfatide (Band I) whereas the more diffuse Band II migrated with slower mobility. When the P-selectin overlay of a TLC plate was prepared in the presence of EDTA, no visible reduction of binding was observed (data not shown). To test whether the Band I corresponds to sulfated-lactosylceramide (SM3), we analyzed cell extracts of HepG2 cells, which are known to contain SM3 sulfatide (Spitalnik, Danley et al. 1989). P-selectin was found to bind the SM3 sulfatide, but this lipid band did not correspond to Band I of MC-38 cells (see Supplemental Material).

Sulfation determines P-selectin ligands on MC-38 cells

To confirm the presence of sulfate groups in the MC-38 lipid bands recognized by P-selectin, cells were metabolically labeled with Sodium ^{35}Sulfate and glycolipids were isolated. Labeled lipid extracts were separated by HPTLC, and the location of the radioactive signal on the plate was determined by film exposure. Sulfate incorporation was detected in the region of Band I as a double band (Figure 3A). The appearance of a double band by metabolic labeling is typical for glycolipids, which indicates a difference in hydroxylation of their acyl chains. However, there was

no signal detected in the region of Band II, indicating that these lipids are not sulfate-containing structures. Indeed, MS/MS analysis of these fractions only detected a series of glycerophosphoinositides in this fraction, in contrast to the same P-selectin binding band from HepG2 cells, where SM2a and SM3 with a short ceramide chain were found, next to the same glycerophosphoinositides (see Supplemental Material). The visualization of glycolipids by orcinol revealed a typical glycolipid pattern as seen before (Figure 2B), while P-selectin staining confirmed the ^{35}S sulfate labeled band to contain selectin ligands (data not shown).

The requirement of sulfation for P-selectin ligand recognition was further confirmed by an inhibition of sulfate metabolism. MC-38 cells were incubated in the presence of sodium chlorate, a known inhibitor of PAPS (3'-phosphoadenosine 5'-phosphosulfate) formation, which is the sulfate donor (Ishizuka 1997). P-selectin binding to sodium chlorate-treated cells was clearly reduced, as detected by flow cytometry (Figure 3B). The extent of P-selectin binding reduction was comparable to arylsulfatase treatment. To confirm that the reduced P-selectin binding to MC-38 after arylsulfatase treatment eliminates the same selectin ligands as achieved by sodium chlorate treatment, MC-38 cells were treated with arylsulfatase followed by sodium chlorate incubation (Figure 3C). The sequential treatment of MC-38 cell did not show any further decrease of P-selectin binding, thereby indicated that both treatments targeted the same ligands. Similarly, the binding of P-selectin to HepG2 cells was reduced by sodium chlorate treatment (see Supplemental Material). All data taken together, we concluded that the Band I P-selectin ligand on MC-38 cells is a sulfated glycolipid.

MALDI-TOF-TOF analysis identified SM4 as P-selectin ligand on MC-38 cells

To characterize the glycolipid recognized by P-selectin on MC-38 cells (Band I in Figure 3A), the LP lipids were treated with phospholipase C and/or were saponified prior to purification on a DEAE-sepharose column. The charged glycolipid fraction was collected and separated by HPTLC. P-selectin immuno-overlay of the plate confirmed the presence of a P-selectin ligand (Figure 4A). The purified samples were analyzed by mass spectrometry as described in Materials and Methods (Figure 4B-E). In non-saponified samples, we detected 2 families of compounds: one family represented the sulfatide SM4, with the heterogeneity contributed by the lipid part (chain length differences, presence of hydroxylation, and saturation levels of the fatty

acid). The slight difference in mobility of Band I, in comparison to SM4 standard as observed by HPTLC (Figure 2C), is possibly due to the total lipid load in the LP lipid–TLC lane. Moreover, a higher fraction of the SM4-ceramide in MC38 cells is hydroxylated as compared to the standard SM4, which typically reduces the mobility under the TLC conditions used here. The other family of compounds was identified as cardiolipin by MS/MS analysis (data not shown). Cardiolipin (CL) has been reported to be virtually exclusively confined to mitochondria, with the exception of apoptotic cells, where it can be found at the plasma membrane (Sorice, Circella et al. 2004). Therefore, it is probably an unlikely candidate for the P-selectin ligand on the surface of cancer cells. Nevertheless, we confirmed *in vitro* that CL is indeed a P-selectin ligand by repeating the overlay experiment with purified cardiolipin from a commercial source (our unpublished results). At this point, we cannot exclude that cardiolipin is also a biological ligand for P-selectin. Upon saponification of the sample, the cardiolipin peaks disappeared from the spectrum, as expected, while the SM4-peaks remained with unaltered distribution. The P-selectin binding signal in the saponified fraction (Figure 4A, lane 3) was rather weak, yet clearly present. These findings indicate that SM4 is P-selectin ligand in MC-38 cells.

MC-38 Cell surface P-selectin ligands are SM4 sulfatides

The identification of SM4 sulfatide in MC-38 cell lipid extracts, together with P-selectin binding to intact cells treated with hydrolytic enzymes (Figure 1), strongly suggested that SM4 sulfatide is the primary P-selectin ligand. To provide further evidence that SM4 sulfatide is indeed the cell surface ligand of P-selectin we analyzed MC-38 cells with altered glycolipid biosynthesis. We have shown that an inhibition of sulfate metabolism led to a decrease of P-selectin binding (Figure 3B). When exogenous SM4 sulfatide was added to untreated cells no alteration of P-selectin binding was observed (Figure 5A). However, the addition of exogenous SM4 sulfatide to sodium chlorate-treated cells almost completely recovered P-selectin binding (Figure 5A). This observation indicated that SM4 sulfatide can reconstitute selectin ligands on MC-38 cells to the same level and binding specificity. To confirm the glycolipid nature of P-selectin ligands, MC-38 cells were incubated in the presence of PPMP, an inhibitor of glucosylceramide synthase. This treatment blocked synthesis of gangliosides and complex sulfatides, only allowing the biosynthesis of galactose-linked glycolipids, where the SM4 sulfatide is the predominant product (Ishizuka

1997). The overall reduction of gangliosides could be detected by orcinol staining of total lipid extracts from equal number of cells, either PPMP-treated or control cells (Figure 5B). The glucosylceramide biosynthesis inhibitor treatment resulted in an enhanced binding of P-selectin to cells (Figure 5A). This increase in P-selectin binding was also observed in the absence of calcium, which was in agreement with the partial calcium independence of P-selectin recognition of a pure SM4 sulfatide (Figure 2A). Finally, trypsin treatment of MC-38 cells did not affect P-selectin binding, while a reduction of PSGL-1 epitopes in a parallel treatment of HL-60 cells was observed (data not shown). Taken together, these results provide evidence that SM4 is the *bona fide* P-selectin sulfoglycolipid ligand on MC-38 cells.

Removal of cell surface sulfation attenuates metastasis

To ascertain whether SM4 sulfatide is the native P-selectin ligand facilitating metastasis, we injected C57Bl/6J mice with MC-38GFP carcinoma cells previously treated with arylsulfatase. Lung sections from mice euthanized 30 min after tumor cell injection were evaluated for platelet-MC-38 interactions (Figure 6A-B). Arylsulfatase treatment reduced the platelet-thrombi formation around tumor cells. Furthermore, platelet-tumor cell emboli formation of MC-38 cells is comparable with previously observed interactions of platelets with human colon carcinoma cells, which carry carcinoma mucins only (Kim, Borsig et al. 1998; Borsig, Wong et al. 2001). Thus, sulfatides on tumor cell surfaces are functional ligands for platelets *in vivo*. When mice were euthanized four weeks later, the extent of metastasis was evaluated (Figure 6C-D). The lungs of mice injected with sham-treated carcinoma cells were almost completely displaced by tumors as observed previously (Borsig, Wong et al. 2002). In contrast, mice injected with arylsulfatase-treated carcinoma cells showed virtually tumor-free lungs. Eight out of ten mice had no or only one visible metastatic foci (Figure 6C). The dramatic reduction of metastasis was further confirmed by fluorescence measurement of the lung homogenate (Figure 6D). To exclude any possibility that the arylsulfatase treatment impaired behavior of cells (Kobayashi, Honke et al. 1994; Uemura, Kabayama et al. 2003), these were analyzed thoroughly *in vitro*. There was no difference in viability, proliferation, adhesion, or morphology of the treated cells when compared to sham-treated cells (data not shown). Heparinase/chondroitinase treated cells behaved as the control cells and exhibited no difference in proliferation, adherence, or morphology (data not shown). Thus it is

unlikely the arylsulfatase treatment affects primarily glycosaminoglycans. Arylsulfatase, but not heparinase/chondroitinase treatment of MC-38 cells also reduced P-selectin-mediated platelet aggregation as observed on lung sections at different time points after intravenous injection. Furthermore, the number of tumor cells trapped in the lung vasculature was lower in mice injected with arylsulfatase treated cells. This agrees with the previously shown decrease of tumor cells seeding to the lungs when selectin ligands on tumor cells were eliminated (Borsig, Wong et al. 2001). The elimination of sulfation on MC-38GFP cells was associated with a reduced platelet aggregation and attenuation of metastasis, confirming the facilitating role of P-selectin in this process.

Discussion

Sulfatides have been detected in various tissues, including brain, kidney and gastrointestinal tract (Ishizuka 1997). A systematic analysis of glycosphingolipids in human gastrointestinal tract has shown the presence of sulfatides, SM4 in particular, in normal mucosa (Natomi, Saitoh et al. 1993). The preferential enrichment of sulfatides in the gastric mucosa strongly indicates its possible involvement in mucosal protection. Sulfatides are also increasingly expressed in several human cancer tissues, including lung (Miyake, Taki et al. 1992), colon (Siddiqui, Whitehead et al. 1978; Osawa, Sugano et al. 1997), kidney (Sakakibara, Gasa et al. 1989), liver (Hiraiwa, Fukuda et al. 1990) and ovary (Kiguchi, Takamatsu et al. 1992). Even though the pathologic correlation of sulfatides with cancer in these tissues remains unclear, their enhanced expression was correlated with poor metastatic prognosis (Morichika, Hamanaka et al. 1996; Makhlouf, Fathalla et al. 2004). Here we provide one possible mechanism for how sulfatides might contribute to metastasis in a syngeneic mouse model. First, SM4 sulfatide was shown to be virtually the sole P-selectin ligand on colon carcinoma cells (MC-38) as determined by MALDI-TOF-TOF analysis of purified glycolipids. Second, P-selectin-mediated interactions with MC-38 cells through SM4 sulfatide were shown to facilitate metastasis, thus identifying SM4 sulfatide to be a functional native ligand in this process. Previously, we and others have shown that the absence of P-selectin led to attenuation of metastasis (Kim, Borsig et al. 1998; Borsig, Wong et al. 2001; Ludwig, Boehme et al. 2004). Here we show that enzymatic removal of sulfation from tumor cells prior to intravenous

injection strongly attenuates metastasis. This observation is in agreement with previous findings that any interference in P-selectin-mediated interactions between tumor cells and platelets and/or endothelium, achieved either by removal of ligands from tumor cells or temporal inhibition by heparin, resulted in attenuation of metastasis, indicating their function in early steps of metastasis (Borsig, Wong et al. 2001; Borsig, Wong et al. 2002; Fuster, Brown et al. 2003; Ludwig, Boehme et al. 2004). Therefore, sulfatides on tumor cells could serve as ligands for P-selectin-mediated interactions, thereby contributing to metastatic spread.

Sulfatides and glycosaminoglycans (e.g. heparin and chondroitin sulfate) represent other non-sialylated ligands for selectins, which are expressed on several carcinomas and also on human granulocytes (Aruffo, Kolanus et al. 1991; Nelson, Cecconi et al. 1993; Suzuki, Toda et al. 1993; Kawashima, Hirose et al. 2000). L-selectin was shown not only to bind SM4 sulfatide, but also sulfated lactosylceramide SM3, and sulfated tri- and tetra-glycoceramides SM2 and SB1a, respectively (Suzuki, Toda et al. 1993). Conversely, P-selectin was demonstrated to bind only SM4 sulfatide (Aruffo, Kolanus et al. 1991). Here we show that P-selectin is also able to recognize SM3, and SM2 sulfatides, which were found in HepG2 cells (Supplementary Material). This observation indicates that a variety of sulfatides can be recognized by P-selectin. Whether sulfatides expressed on variety of human carcinomas (see above) contribute to metastatic spread through selectin mediated interaction requires further studies. Interestingly, sulfatides were found to facilitate P-selectin-mediated platelet adhesion and aggregation (Merten and Thiagarajan 2001). The recent finding that sulfatides could lead to platelet activation and their aggregation with leukocytes suggest this mechanism to play an important role in hemostasis and thrombosis (Merten, Beythien et al. 2005). In this context, our finding that murine colon carcinoma cells carrying sulfatides can effectively induce P-selectin-mediated platelet aggregation raises the possibility for the involvement of these interactions also during hematogenous metastasis.

The negatively charged phospholipid cardiolipin (CL) is normally confined to the mitochondrial inner membrane (Schlame, Rua et al. 2000). In addition, CL was also detected on the cell surface of apoptotic cells after redistribution from mitochondria (Sorice, Circella et al. 2004). Modulation of the internal CL pool by palmitate was associated with apoptosis of breast cancer cells (Hardy, El-Assaad et al. 2003). We have shown that CL can be purified from membranes of MC-38 cells and

is recognized by P-selectin. By HPTLC separation CL was co-migrating with SM4 sulfatide and could be only eliminated by saponification. Interestingly, significant amounts of CL were found in MC-38 cell extracts, while CL usually comprises a minor component of the total lipid extracts in other cell lines (our unpublished results). Previously, L-selectin was shown to bind CL in calcium-independent manner (Malhotra, Taylor et al. 1996). We have confirmed that P-selectin can bind CL. Whether the enhanced presence of CL in MC-38 has any biological relevance for P-selectin recognition remains to be elucidated. Nevertheless, MC-38 cells after sulfatase treatment or inhibition of sulfate metabolism by sodium chlorate showed significantly reduced P-selectin binding, strongly suggesting that the main ligand for P-selectin-mediated metastasis is a sulfoglycolipid.

The association of enhanced sulfatide expression on cancer cells with metastasis has not been fully clarified. Human renal-cell carcinoma and hepatocellular carcinoma cells require cell surface expression of sulfoglycolipids for attachment to laminin (Kobayashi, Honke et al. 1994; Zhong Wu, Honke et al. 2004). Incorporation of externally supplemented SM4 sulfatide increased haptotactic migration of cancer cells on laminin, which was associated with accelerated lung colonization (Kobayashi, Honke et al. 1994). The presence of sulfatides on cancer cells was correlated with the expression of ceramide sulfotransferase gene (CST) (Honke, Tsuda et al. 1998) and associated with metastatic potential of human hepatocellular carcinoma cells (Zhong Wu, Honke et al. 2004). Expression of particular sulfatides was also found to be dependent on the availability of precursor glycolipids (Kobayashi, Honke et al. 1994; Zhong Wu, Honke et al. 2004). Meanwhile the presence of SM3 sulfatide was linked to up-regulation of CST gene expression, which also mediated cell adhesion to vitronectin and $\alpha V\beta 3$ integrins (Honke, Tsuda et al. 1998; Zhong Wu, Honke et al. 2004). On the contrary, expression of SM3 sulfatide induced by CST transfection in murine Lewis lung carcinoma cells led to suppression of cell adhesion to laminin and $\beta 1$ integrin, and subsequently metastasis (Kabayama, Ito et al. 2001; Uemura, Kabayama et al. 2003). The discrepancy between these results may be due to the clonal nature of the Lewis lung carcinoma cells and the subsequent differences in their signal transduction (Kabayama, Ito et al. 2001; Uemura, Kabayama et al. 2003). Recently, sulfatides were also found to be potential native ligands for Galectin-4, a galactose-binding lectin (Ideo, Seko et al. 2005). Our findings propose that SM4

sulfatide is contributing to metastasis through P-selectin mediated interactions with platelets and/or endothelium. The extent of metastasis observed with MC-38GFP cells devoid of sulfation, and thereby P-selectin ligands (Figure 6), was even less than that observed in P-selectin deficient mice (Borsig, Wong et al. 2002). Thus it is likely that sulfatides may contribute to metastasis by more than one mechanism. Further studies are needed to elucidate how P- and/or L-selectin binding to sulfatides affect metastasis.

Materials and Methods

Cell lines and reagents

Mouse colon carcinoma cell line MC-38 (Borsig, Wong et al. 2002) was grown in DMEM with high glucose (10% FCS) medium (Invitrogen). For experimental metastasis studies, we used cells stably expressing GFP (MC-38GFP) (Borsig, Wong et al. 2002). All reagents were from Sigma unless otherwise stated.

Flow cytometry analysis of MC-38 cells

Cells were grown to 90% confluency, detached by incubating in PBS containing 2mM EDTA for 5 min at 37 °C and washed three times with cold Hank's Balanced Salt Solution (HBSS). After blocking with HBSS containing 1% BSA (HBSS/BSA), cells were incubated with mouse P- or L-selectin chimeras, containing the Fc region of human IgG (Borsig, Wong et al. 2002). Prior to addition to tumor cells, selectin chimeras were pre-incubated with a biotinylated goat-anti-human IgG Ab (1:200) for 1 h at RT in HBSS/BSA. After incubating tumor cells with pre-conjugated selectin chimeras for 1 h at 4 ºC, Streptavidin-PE-Cy5 (Becton Dickinson) was added and incubated for 20 min. Cells were washed with HBSS/BSA and analyzed by flow cytometry. As controls, cells were stained with P-selectin chimera in the presence of 5 mM EDTA and 30 mM EDTA, respectively (Borsig, Wong et al. 2002). In some cases, tumor cells were enzymatically treated prior to incubation with selectin chimeras. Glycosaminoglycans were removed by treatment with a mixture of heparinase II and chondroitinase ABC in HBSS for 1 h at 37 ºC (Borsig, Wong et al. 2002). Sulfate groups were eliminated by Arylsulfatase H-5 treatment in HBSS buffer for 1 h at 37 ºC.

MC-38 cells adhesion to immobilized platelets

Mouse platelets were isolated and labeled with Calcein AM as previously described (Kim, Borsig et al. 1998). Glass chamber slides (LabTek, Nunc) were pre-treated with 4% solution of 3-aminopropyltriethoxysilane (Buttrum, Hatton et al. 1993). Isolated platelets were added to the chamber slides and spun at 200g for 10 min. Slides were blocked by PBS/BSA for 10 min. Platelets were activated with thrombin (1U/ml) for 10 min. Even coating of the slide by platelets was confirmed by fluorescence microscopy. Slides coated with platelets were loaded with 20'000 MC-38 cells/well and incubated for 20 min on Orbital shaker at 70 rpm (Koenig, Norgard-Sumnicht et al. 1998). In some cases, P-selectin expressed of platelets was blocked by an addition of 6μg of function-blocking P-selectin antibody (BD Pharmingen). After incubation non-attached cells were aspirated and slides were washed once with PBS. Attached MC-38 cells were counted at the microscope 12 view fields by x100 magnification.

Lipid extraction from MC-38 and HepG2 cells

Semi-confluent cells were detached with PBS containing 2 mM EDTA and washed three times with HBSS. Cells were resuspended in water and homogenized by sonicating three times for 1 min. Lipids were extracted with 10 volumes of chloroform/methanol (1:1) for 4 h at RT while shaking. Supernatants containing lipids were separated by centrifugation and the cell pellet was re-extracted with chloroform/methanol (1:1) overnight. Pooled lipid-rich supernatants were separated by Folch partition (Schnaar 1994) where two phases were obtained: the upper phase containing less hydrophobic and the lower phase containing more hydrophobic lipids. The upper phase was dialyzed against water. The lower phase was treated with phospholipase C (PLC) in 50 mM potassium phosphate buffer, pH 7.4 (Sonnenburg, van Halbeek et al. 2002), and incubated at 37 ºC for 3 h while vigorously shaking, followed by dialysis against water and drying down. The sugar content of the lipid extract was measured by a phenol-sulfuric acid colorimetric assay (Dubois, Gilles et al. 1956). Briefly, an aliquot of the lipid preparation or various concentrations of glucose were added to a glass vial, and brought to 200 μl with water. Phenol was added (to 5% final concentration) together with 1ml of concentrated sulfuric acid. Probes were vigorously mixed and the absorbance was measured at 490 nm. Sugar content of the lipid extract was calculated against a standard curve obtained from the various glucose solutions.

ELISA analysis of lipids from MC-38 cells

Lipids, along with bovine sulfatide (SM4) as a control, were resuspended in methanol and used for coating of the ELISA plates by evaporating the methanol (Borsig, Wong et al. 2002). The wells were washed with HBSS and blocked with 0.5% BSA in HBSS for 1 h at RT. Mouse selectin chimeras were pre-incubated with goat-anti-human IgG Ab conjugated with alkaline phosphatase (1:100) for 1 h at RT. Preincubated selectins were added into the wells and incubated for 2 h at RT in the absence or presence of 30 mM EDTA, followed by three washes with HBSS. After a final wash, the p-nitrophenyl phosphate substrate was added and allowed to develop for 20 min at RT, after which absorbance was measured at 405 nm. Some lipid samples were enzymatically treated prior to coating on ELISA plates (Hiraiwa, Fukuda et al. 1990). Briefly, aliquots of lipid fractions were placed in glass vials and dried down under a N_2 stream. Samples for Arylsulfatase H-5 treatment were resuspended in 50 mM acetate buffer, pH 5.0, while those for neuraminidase treatment were treated in 50 mM citrate buffer, pH 5.0. After treating for 90 min at 37 ºC, lipids were dried down and resuspended in methanol.

HPTLC (high performance thin layer chromatography)

Fifteen μg of glucose equivalents of our sample were applied to a pre-activated silica plate (MERCK). Plates were developed in a TLC tank pre-equilibrated with chloroform/methanol/0.25% $CaCl_2$ (50:40:10) or with chloroform/methanol/0.2%$CaCl_2$ (60:35:7). SM4 and a neutral ganglioside mix (Matreya) were used as markers. Glycolipids were visualized by the orcinol-sulfuric acid reagent.

TLC immuno-overlay

Samples were applied to TLC plates and developed as described above. Dried plates were plastified in a solution of Hexane/2% polyisobutylmethacrylate (ALDRICH) in chloroform (84:16) (Schnaar 1994). Air-dried plates were incubated with a blocking solution, HBSS containing 5% BSA, for 2 h at RT followed by incubation with P-selectin chimera (Borsig, Wong et al. 2002) in HBSS/BSA overnight at 4 °C. Plates were washed three times with HBSS/BSA and incubated with a secondary antibody

conjugated with horseradish peroxidase for 1 h at RT. After three washes with HBSS, plates were developed with 400 μg/ml o-phenylendiamine in 50 mM citrate-phosphate buffer, pH 5.0, with 0.12% hydrogen peroxide and dried (Sonnenburg, van Halbeek et al. 2002).

Metabolic ^{35}S labeling of lipids

MC-38 cells were grown in T175 flasks until 80% confluent. The complete medium was changed for a low sulfate medium containing Met (7.5 mg/l) and Cys (50 mg/l) supplemented with 10% dialyzed FCS. After sulfate starving for 3 h, cells were labeled with 0.5 mCi of Sodium $[H^{35}SO_4]^-$ in the low sulfate medium for 16 h (Tooze 2003). Labeled cells were washed with PBS and detached with PBS/2mM EDTA. Total lipids were extracted from the cells as follows: the cell pellet was resuspended in five volumes of H_2O, vortexed and bath sonicated followed by an addition of 10 volumes of chloroform/methanol (1:1). Lipids were extracted for 3-4 h at RT while shaking vigorously. Samples were centrifuged at 4 °C to pellet the precipitates. The supernatant was transferred into a new tube and stored at 4°C. The pellet was re-extracted overnight in a similar volume of chloroform/methanol (1:1). Both lipid extractions were pooled and dried down. Samples were resuspended in methanol, loaded on a TLC plate and developed as described above. Lipids, which incorporated sulfate, were revealed by exposure to X-ray film (Kodak) for 15 days. The TLC plate was stained with orcinol-sulfuric reagent.

Purification of sulfoglycolipids through anion exchange chromatography (DEAE-Sepharose) and preparative HPTLC

To hydrolyze triacylglycerides, glycerophospholipids, and sphingolipids, the lower phase was saponified with ammonia solution according to an adopted protocol (Ferguson 1993). The samples were dried down and resuspended in a solution containing a final concentration of 17.5% ammonia in methanol. After incubation for 6 h at 50 ºC, the preparation was dried down in a SpeedVac centrifuge and washed twice with 50% methanol. Separation of negatively charged lipids from neutral lipids was performed as described previously (Schnaar 1994). Briefly, PLC-treated or saponified lower phase lipid extract was dissolved in solution A, chloroform/methanol/water (30:60:8 v/v). The DEAE-sepharose (Amersham

Biosciences) was changed into acetate form by mixing the dry resin with solution B, chloroform/methanol/0.8M aqueous sodium acetate (30:60:8 v/v). After washing the column several times with solution A, the samples were loaded and the flow through was discarded. The negatively-charged lipids were eluted with several bed volumes of solution B. The eluate was dialyzed and samples were separated by HPTLC. The areas corresponding to lipids recognized by P-selectin were scratched and collected into a glass tube. To recover the lipids from silica, samples were resuspended in methanol, mixed and spun down. The supernatant, containing the lipids, was transferred into a new tube and dried down.

Modulation of cellular sulfatides

MC-38 cells and HepG2 cells were incubated in regular medium containing 100 mM sodium chlorate for 72 h (Xia, Ramachandran et al. 2003). Cells were detached by 5 mM EDTA/PBS treatment and the extent of P-selectin binding was analyzed by flow cytometry. When the incorporation of externally added SM4 sulfatide was evaluated, cells were resuspended in HBSS containing 1% BSA at concentration of 2 x 10^6 cells/ml and were incubated for 5 min with 10 μM SM4 sulfatide at 37°C (Kobayashi, Honke et al. 1994). Cells were immediately put on ice, washed with ice-cold HBSS buffer and incubated with P-selectin as previously described. For the inhibition of glucosylceramide synthase, tumor cells were cultured in the presence of 15 μM of *PPMP* (D,1-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-propanol; Matreya) (Burdick, McCaffery et al. 2003; Dimitroff, Lechpammer et al. 2004). After 72 h cultivation in the presence of the inhibitor, cells were stained with P-selectin and analyzed by flow cytometry.

Mass spectrometry

For MALDI-TOF and TOF/TOF mass spectrometry, preparative TLC was performed as described above. Upon solvent development, the aluminum-backed plate was cut through the lane of interest. One half was used for detection of the regions of interest using P-selectin immuno-overlay, upon which both halves of the plate were re-aligned, and the silica present in narrow bands on the non-stained half of the plate were scraped off and carefully collected. After having experienced significant losses of analytes on plastics and chromatographic materials, we found that simple

extraction of the silica with 100% methanol yielded preparations that were sufficiently pure for direct analysis via MALDI-TOF MS, while minimizing the chance for material loss during sample workup. We used 192-spot polished stainless steel targets for the ABI 4700 Proteomics Analyser. Small droplets of the methanol-extracts were applied onto the target spot under a continuous stream of cold air, to allow instantaneous drying. We repeated this sample application procedure until a whitish film became visible on the dried target spot (applying more sample results in decreased spectral quality). We used a preparation of 20 mg/ml 6-aza-2-thiothymine (ATT; Fluka, Buchs, CH) in 70% MeOH containing 10 mM NH_4-citrate for the matrix. One microliter of this matrix preparation was spotted onto the sample-loaded target spots and pipetted up and down to provide for proper sample-matrix mixing. The drops were then left to dry. ATT was chosen at the matrix by comparing this matrix for analysis of the SM4 standard with several other preparations that have been reported in the literature to be useful for polar lipids (2,5-dihydrozybenzoic acid, 2,5-dihydroxybenzoic acid/5-methoxysalicylic acid comatrix, 3-hydroxypicolinic acid/6-hydroxypicolinic acid co-matrix, α-hydroxy-4-cyanocinnamic acid). The ATT matrix is exceptionally 'clean' in the low-m/z region which is important for the analysis of lipids, and is sufficiently 'cold' to allow for intact ionization of sulfated, mono- and di-sialylated, and phosphorylated lipids. All the reported analyses were performed in the negative ion reflectron mode of the instrument, tuned for an optimal sensitivity/resolution ratio for this matrix. Calibration in the 900-4000 m/z range was performed using a standard peptide mixture (ABI) in the same ATT matrix as used for the lipid analytes. The spectra were obtained by summation of at least 50 subspectra, each averaged over 50 laser shots. TOF/TOF MS/MS analysis was carried out with air at 5.10E-6 Torr as the collision gas, and the spectra were obtained by summation of 100 subspectra, each averaged over 50 laser shots. The instrument was optimized to select the parent ion ± 3-4 Da, which is the narrowest available limit with this ion selector.

Methodological note: a mass spectrometry method to distinguish sulfation from phosphorylation

Sulfation and phosphorylation of a certain class of biomolecules (in this case polar lipids) can be very difficult to distinguish at trace levels. In the analytical situation we faced here, sulfoglycolipids were present in a mixture with phospholipids and, most

problematically with phosphoinositides. To obtain positive evidence for sulfation versus phosphorylation, we made use of the fact that TOF/TOF fragmentation follows the behavior of higher-energy single collision-induced fragmentation as is typical for triple quadrupole and magnetic sector instruments, rather than the behavior of low-energy multiple collision-induced fragmentation as is observed in most of the 'modern' analyzers (ion traps). In high-energy collision induced dissociation (CID), sulfated compounds yield the sulfite radical anion $[\cdot SO_3]^-$ at m/z 80 and $[HSO_4]^-$ at m/z 97. Phosphorylated compounds yield $[PO_3]^-$ at m/z 79 and $[H_2PO_4]^-$ at m/z 97 (Wilm, Neubauer et al. 1996). We report here that MALDI-TOF/TOF yields these diagnostic high-energy CID ions very reliably and at sufficiently high intensities to be potentially useful also in phospho- versus sulfo-proteomics. We suspect that the extremely short time between CID and detection in the TOF/TOF geometry contributes to the radical anion being robustly detected. As Ser/Thr sulfation has recently been described in a wide range of organisms, and as the distinction between Ser/Thr sulfation and phosphorylation cannot be made using the most common 'phosphoproteomics' methodology, the TOF/TOF analyzer will be extremely useful for this purpose.

Experimental metastasis assays

Mice (strain C57Bl/6J) were injected with 3-4x 10^5 MC-38GFP syngeneic adenocarcinoma tumor cells, which were either arylsulfatase or mock treated. After four weeks, mice were euthanized, a picture of dissected lungs was taken and lungs were further processed for quantitation of metastasis by detection of GFP fluorescence (Borsig, Wong et al. 2002)

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Abbreviations

CL, cardiolipin; CST, cerebroside sulfotransferase; HPTLC, high performance thin layer chromatography; MALDI TOF/TOF, Matrix-assisted laser desorption/ionization

time-of-flight (mass spectroscopy); PLC, phospholipase C; SM2, sulfated triganglioside GalNAcβ-4(HSO_3-3)Galβ-4Glcβ-1Cer; SM3, sulfo-lactosylceramide HSO_3-3Galβ-4Glcβ-1Cer; SM4, sulfated galactosylceramide/ sulfatide HSO_3-3Galβ-1Cer.

Figure Legends

Fig. 1. *Characterization of the nature of selectin ligands on MC-38 cell surfaces.* (**A**) MC-38 cells were stained with mouse selectins and analyzed by flow cytometry. (**a, c**) P-selectin and (**b, d**) L-selectin stained untreated cells are represented by bold solid lines. (**a, b**) Filled areas represent controls stained with the secondary Ab only. The dashed line represents selectin stained cells after heparinase-chondroitinase treatment, while the thin line represents selectin stained cells after arylsulfatase treatment. (**c, d**) Thin line represents selectin staining in the presence of 30mM EDTA. (**B**) Adhesion of MC-38 cells to immobilized platelets. Arylsulfatase-treated or sham-treated MC-38 cells were incubated on slides with activated platelets for 20 min under low shear. Adherent cells were counted.

Fig. 2. *Lipid extracts of MC-38 cells contain P-selectin ligands.* (**A**) ELISA plates were coated with LP lipids isolated by Folch partitioning (white bars) and with bovine sulfatide-SM4 (black bars) as a control. *No treatment*- represents P-selectin binding to extracted lipids. *EDTA*- represents P-selectin binding to lipid fraction in the presence of 30 mM EDTA. The third and fourth sets show P-selectin recognition of lipids after enzymatic treatment, desulfation or desialylation, respectively. Data shown are mean ± SD of triplicates. The difference between non-treated lipids and arylsulfatase treated lipids (both MC-38 extracts and SM4 sulfatide) were statistically significant; as determined by one-way ANOVA followed by a pair wise t-test, $p< 0.001$. (**B**) and (**C**), MC-38 lipid extracts were separated by HPTLC and developed in Chloroform:methanol:0.2%$CaCl_2$ (60:35:7). Loading of samples: SM4 (lane 1); LP lipids from MC-38 cells (lane 2); UP lipids of MC-38 cells (lane 3) and a neutral ganglioside marker (lane 4). (**B**) Glycolipids visualized by orcinol-sulfuric acid staining. (**C**) P-selectin overlay of separated lipid ligands.

Fig. 3. *P-selectin ligand recognition requires sulfation.* (**A**) MC-38 cells were metabolically labeled with ^{35}S sodium sulfate as described in Materials and Methods.

Lipid extracts were separated by HPTLC and the incorporation of the radio-labeled sulfate was detected by exposure to X-ray film with a subsequent visualization by orcinol-sulfuric acid staining. (**B**) MC-38 cells were incubated in the presence of 100 mM sodium chlorate for 72 h before staining with P-selectin and flow cytometry analysis. Bold line represents cells treated with sodium chlorate; shaded area represents cells without treatment; thin line represents cells with arylsulfatase treatment only. (**C**) P-selectin staining of MC-38 cells. **a –** MC-38 cells were treated with arylsulfatase (bold line) and compared to sham-treated cells; **b –** Arylsulfatase treated cells were further incubated for 72 h in the presence of sodium chlorate (thin line). Bold line represents sham-treated cells (panel a), which were incubated in sodium chlorate. Shaded area represents sham-treated cells incubated in normal medium as a control.

Fig. 4. *MALDI TOF and TOF/TOF mass spectrometry analysis of the MC-38 P-selectin ligand.* The specific band recognized by P-selectin was extracted from the TLC plate and prepared for mass spectrometry analysis. (**A**) Immuno-overlay of the TLC-separated lipids with P-selectin. MC-38 lipid extracts from Folch partionings lower phase treated with PLC (lane 2) or saponified (lane 3) and purified on a DEAE column; SM4 sulfatide standard (lane 1). (**B**) Negative reflectron mode profile of Band I recognized by P-selectin in MC-38 lipid extract treated with phospholipase C. There are two families of peaks: around 890 m/z, corresponding to SM4, and a second group about 1400-1475 m/z that corresponds to cardiolipin. *Insert* corresponds to SM4 region. (**C1**) Negative reflectron mode profile of Band I recognized by P-selectin in MC-38 lipid extracts saponified with ammonia as described in Materials and Methods. *Insert* corresponds to SM4 region. Cardiolipin is undetectable upon this saponification procedure. (**C2**) MS/MS negative ion mode profile of m/z 890.6. The fragment at 80 (m/z) represents the sulfate-specific radical anion $[\cdot SO_3]^-$, while the fragment at 97.1 m/z represents $[HSO_4]^-$. Together, this ion fragment pair is diagnostic for sulfated compounds under these conditions. The peaks at 241.1 and 259.1 m/z correspond to the B_1- and C_1- fragments, respectively, resulting from cleavage of the glycosidic bond which links the hexose-sulfate moiety to the ceramide. *Insert* corresponds to the region 65-300 m/z. (**D1**) Negative reflectron mode profile of SM4 standard after being purified by TLC as described in Materials and Methods. *Insert* corresponds to SM4 region. A different pattern of lipid heterogeneity of the SM4 spectra from MC-38 cells versus the standard could be observed. (**D2**) MS/MS

negative ion mode profile of m/z 888.5 in the standard. *Insert* corresponds to the region 65-300 m/z. Note the virtual identicalness of the fragment ion pattern between the standard SM4 and the compound in panel (**C2**), further confirming the identity of this compound as SM4.

Fig. 5. *Modulation of glycolipid biosynthesis affects P-selectin binding.* (**A**) MC-38 cells were stained with mouse P-selectin and analyzed by flow cytometry. (**a**) MC-38 cells exposed to exogenous SM4 sulfatide did not alter P-selectin binding to cells (Bold line); Filled area represents control cells without sulfatide addition; (**b**) To sodium chlorate treated MC-38 cells (bold line) was exogenously added SM4 sulfatide and the cells were stained with P-selectin (thin line); Filled area represents binding of P-selectin to untreated cells. (**c**) MC-38 cells were incubated in presence of PPMP for 72 h and stained with P-selectin (bold line). P-selectin binding was only partially affected when incubated with 30 mM EDTA (thin line); Control cells, without glucosylceramide synthase inhibitor, were stained with P-selectin without EDTA (filled areas), or in the presence of EDTA (dotted line). (**B**) Total lipid extracts from cells treated with PPMP (PPMP) or untreated (control) were separated by HPTLC and stained by orcinol. Equal amount of cells has been extracted and loaded on TLC plate. Reduction in monosialogangliosides as well as highly charged glycolipid could be observed.

Fig. 6. *Elimination of cell surface sulfation attenuates metastasis.* Mice were injected intravenously with 3-4 x 10^5 MC-38GFP cells either treated with arylsulfatase or mock control. (**A**) Platelet interactions with injected cells were evaluated in lungs from mice terminated 30 min after injection. Representative images untreated-control tumor cells (green) with associated platelets (red), which were largely diminished when MC-38 cells were treated by arylsulfatase prior to injection. Blue are stained muclei. (**B**) Quantification of platelet-tumor cell interactions from 3 independent experiments. (**C**) and (**D**) Mice were euthanized after 28 days, lungs were dissected and photographed, and GFP fluorescence in the lung homogenate was quantified. The number of animals studied was 8-9 in each group. (**C**) Representative examples of dissected lungs from mice injected with arysulfatase treated (+) and mock-treated (-) MC-38GFP cells. (**D**) The extent of metastasis was measured by GFP fluorescence analysis of lung homogenate. Statistical significance was determined by Student's t-test.

Figure 1

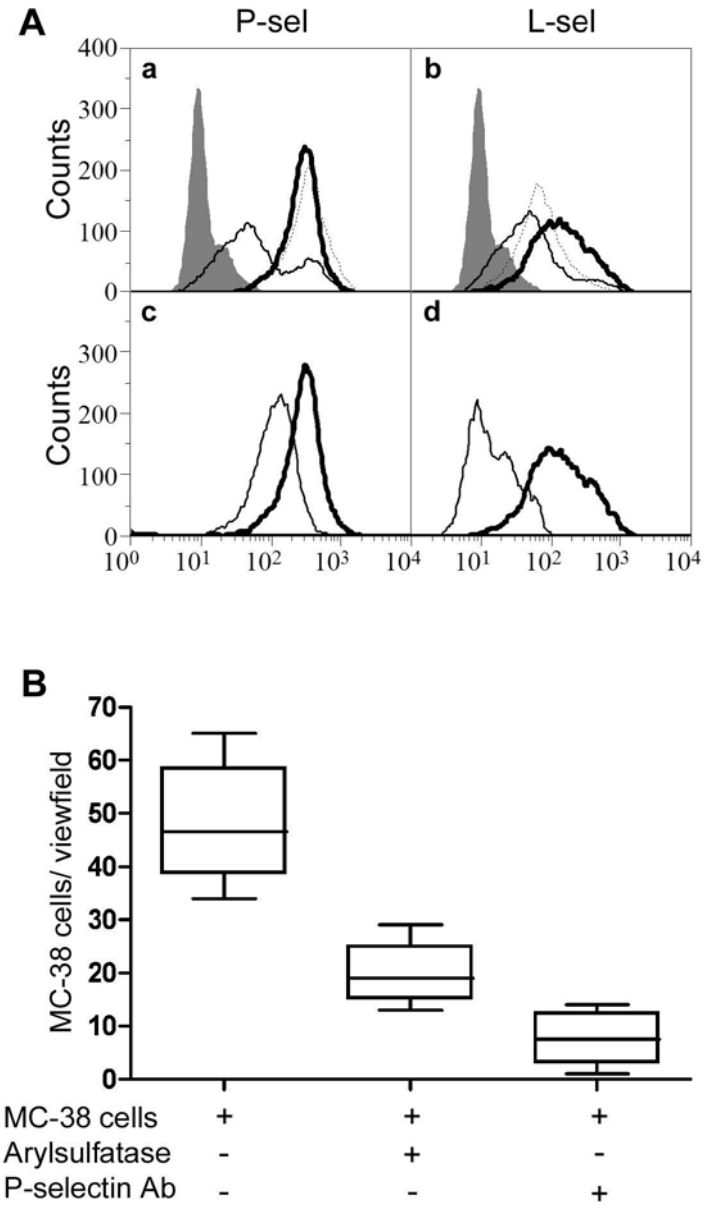
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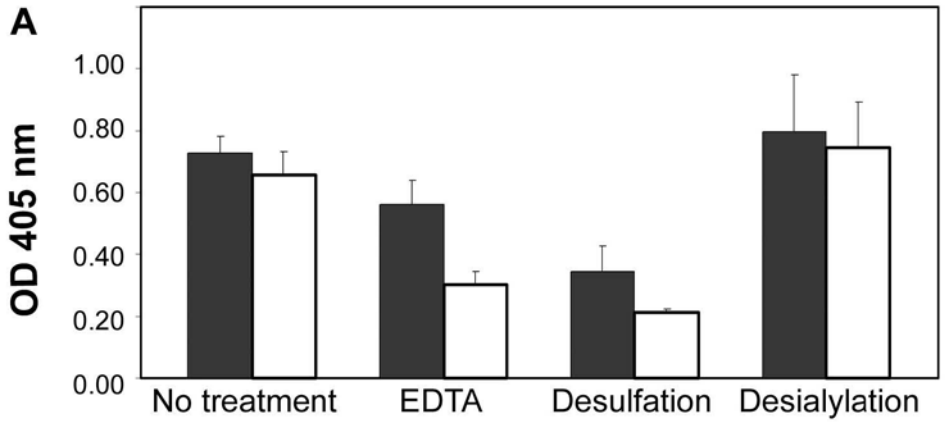
Figure 2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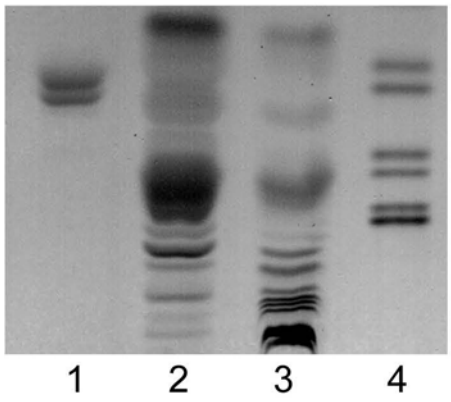
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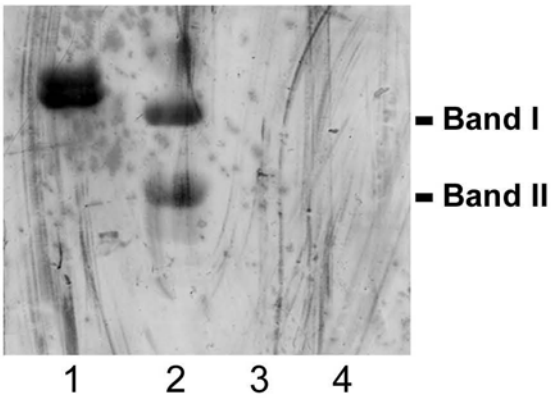
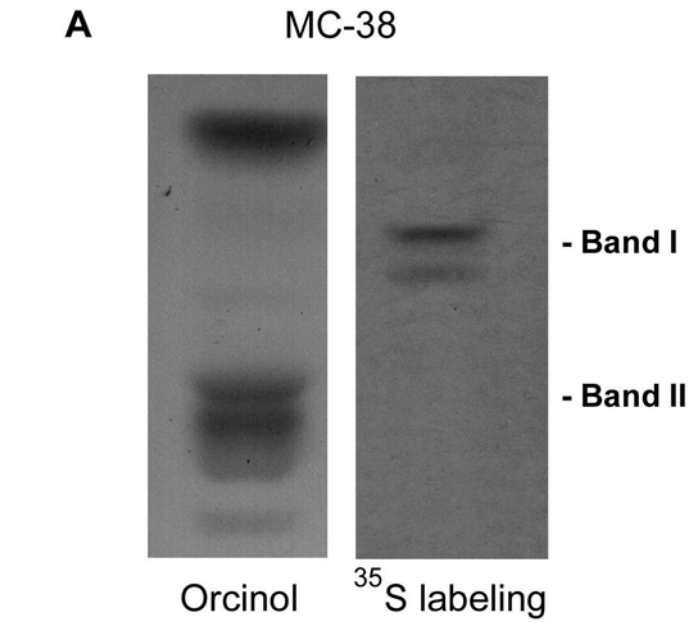

C
Band I
Band II
1
2
3
4

Figure 3


A
MC-38
- Band I
- Band II
Orcinol
35S labeling

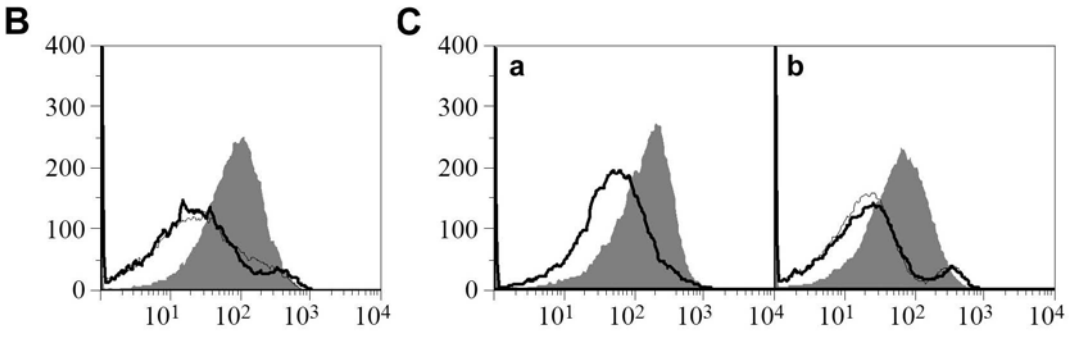

B
C
400
300
200
100
0
a
b
10¹
10²
10³
10⁴

Figure 4

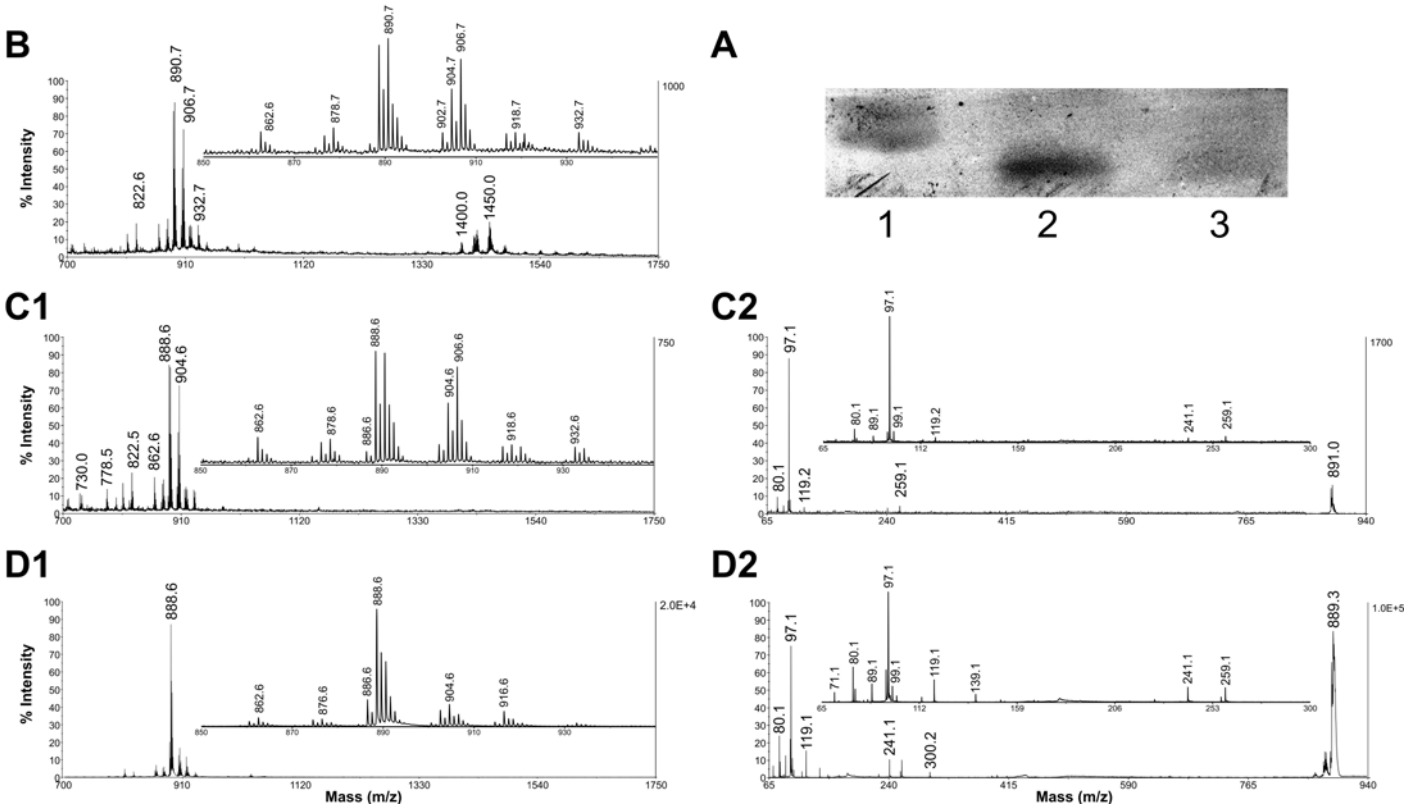


Figure 5

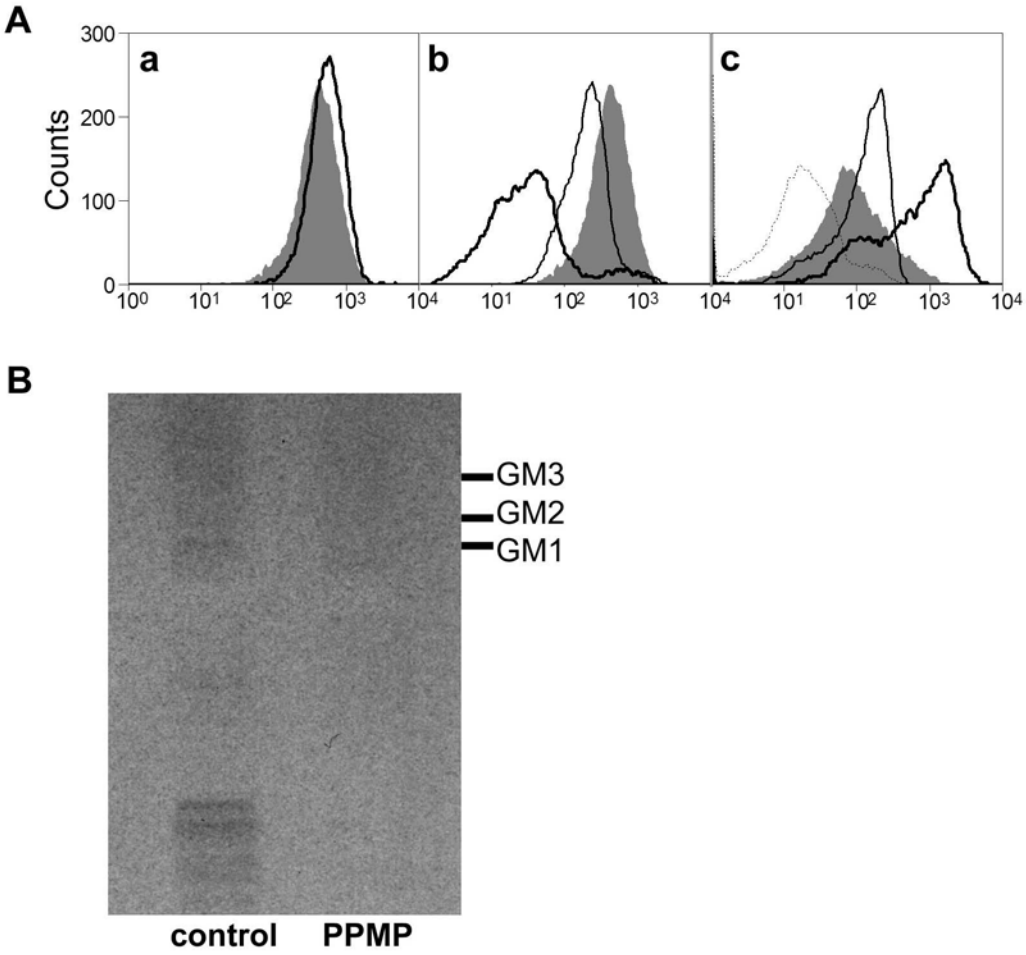
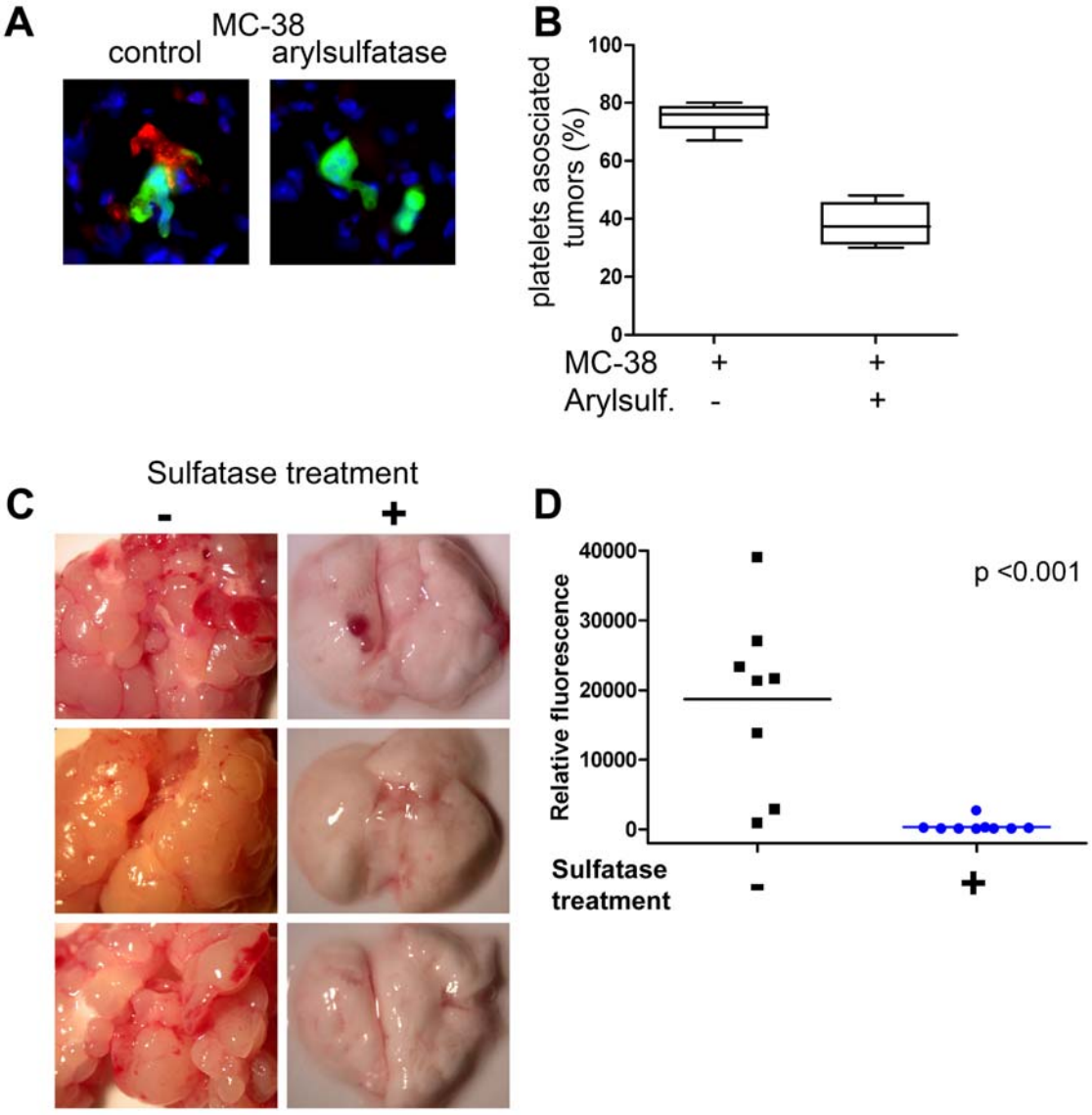

A
a
b
c
Counts
300
200
100
0
10^0 10^1 10^2 10^3 10^4
B
GM3
GM2
GM1
control
PPMP

Figure 6


A
MC-38
control
arylsulfatase
B
platelets asosciated tumors (%)
100
80
60
40
20
0
MC-38 + +
Arylsulf. - +
C
Sulfatase treatment
-
+
D
Relative fluorescence
40000
30000
20000
10000
0
p <0.001
Sulfatase treatment
-
+

Supplementary Material

P-selectin ligands on HepG2 cells are also sulfated glycolipids.

Flow cytometry analysis of HepG2 cells showed that P-selectin binding was partially calcium independent and sensitive to arylsulfatase treatment, indicating sulfatides as the ligands (Fig. S1A). Heparinase-chondroitinase treatment of cells had little effect on P-selectin binding. Total lipid extracts were separated by Folch partition and the phases were analyzed by HPTLC developed in Chloroform/Methanol/0.25% CaCl2 (50:40:10). Figure S1B shows SM4 sulfatide standard (lane 1) and the LP of HepG2 cell lipid extract (lane 2). P-selectin immuno-overlay revealed three distinct bands. Band I and Band II corresponded to lipids identified on MC-38 cells. There was a third band (Band III) detected between Band I and II according to its mobility. The immuno-overlay with 7-E10 Ab (kind gift of Prof. Kannagi) specific for SM3 sulfatide identified the Band III as SM3 sulfatide. Glycolipids were visualized by orcinol-sulfuric acid staining. When HepG2 cells were metabolically labeled with Sodium ^{35}Sulfate, glycolipids were isolated and separated by HPTLC. Sulfate incorporation was detected in all 3 bands recognized by P-selectin (Fig. S1C).

Figure S2 shows MALDI TOF and TOF/TOF MS analysis of glycolipids from HepG2 cells recognized by P-selectin. Three bands recognized by P-selectin were extracted from the TLC plate and prepared for mass spectrometry analysis. In the following MS profiles, only the peaks which were found in MS/MS analysis to correspond to sulfatides are highlighted in bold. A1, Negative reflectron mode profile of Band I recognized by P-selectin in HepG2 lipid extract treated with phospholipase C. Only the peak around 890 m/z corresponds to sulfatide SM4, as confirmed through MS/MS in panel A2 (fragments at m/z 80, 97 and 241). The analytes at 746.5, 773.5 and 938.5 m/z are glycerophospholipid family members (as attested by an m/z 153 fragment in MS/MS mode, data not shown), and the analytes at 1400-1475 m/z correspond to cardiolipin (see main text). B1, Negative reflectron mode profile of Band III recognized by Pselectin in HepG2 lipid extracts treated with phospholipase C. Analytes at 940.6 and 1066.7 m/z show a virtually identical fragmentation pattern (panels B2 and B3, respectively), with sulfate-diagnostic ion pair at m/z 80.0 and 97.0, and with ion pairs at 241.1 (B1) and 259.1 (C1), and at 403.1 (B2) and 421.1 (C2), indicative of glycosidic bond cleavage in a dihexose structure with a sulfate

moiety on the non-reducing monosaccharide. This fragmentation behavior is compatible with the sulfolactose headgroup structure characteristic of SM3 sulfatides. Inset corresponds to the region 65-500 m/z. The fragmentation pattern of the sulfated headgroup is also identical to that of the compound at 956.5 m/z in the Band II preparation (Panel C1 and C3). Differences in mass between these SM3 family members are likely due to differences in the ceramide structure. A difference of 110 Da between glycosphingolipids of the same family has been previously observed (1) and was explained as a C8H14 moiety containing 1 unsaturation. The extra difference of 16 Da between the SM3 compounds in Band III can easily be explained as a ceramide hydroxylation on the larger one. One would thus conclude that the gain in hydrophobicity of a ceramide hydroxylation is offset by 8 carbon-atom shorter hydrophobic chain length, as these compounds have very similar mobilities in our TLC separation. The SM3 family member both with a short chain and a hydroxylated ceramide thus is more hydrophilic and consequently runs together with SM2, which differs from SM3 by an extra GalNAc residue. C1, Negative reflectron mode profile of Band II recognized by P-selectin in HepG2 lipid extracts treated with phospholipase C. The compound at 956.5 was already described above. The peak at m/z 1269.7 corresponds to yet another sulfatide (diagnostic fragment ion pair at 80 and 97 m/z).

Interpretation of its MS/MS spectrum is somewhat more complicated, however. We observe the Y3 fragment at m/z 1066.7, corresponding to a loss of a HexNAc residue (203 Da). The resulting mass corresponds to the SM3 mass observed in panel B1, which, together with the knowledge that it concerns a sulfated lipid, indicates that the structure resulting from this fragmentation corresponds to the hydroxylated long-ceramide chain SM3. Based on the biosynthetic pathway for sulfatides in mammalian cells, it is thought that SM2a (GalNAc-substituted SM3) is the likely interpretation. This is confirmed by the detection in negative ion mode MS/MS of the B3 fragment at m/z 606.0. In positive ion mode MS/MS of the ion at 1315 m/z (disodiated adduct of 1269.7) we likewise observe the ion pair at 651.9 and 669.9 m/z (see inset of Panel C2), corresponding to the B3 and C3 fragments that one would predict from the disodiated SM2a species (cleavage at the glycosidic bond linking the entire sulfotrisaccharide headgroup to the ceramide moiety). The interpretation of this analyte as SM2a is also compatible with the lack of fragment

ions at m/z 241/259 and 403/421 in negative ion mode MS/MS, as the sulfate moiety is subterminal in this structure.

The other analytes in Bands II and III correspond to glycerophospholipids of the phosphoinositide family. Band II from MC38 cells (see main text) has the same mobility as HepG2 Band II. The MS/MS spectrum of the compound in HepG2 Band II at 887.6 is shown in Panel C4. One detects an ion pair at 79 ($[PO3]^-$) and 97 ($[H2PO4]^-$) m/z (in contrast to 80 and 97 m/z for sulfated species under these TOF/TOF conditions), the glycerophospholipid signature ion at 153 m/z ($[glycerolphosphate\text{-}H2O]^-$), the inositolphosphate fragment at 241.0 and fatty acid ions at 283.3 and 305.3 m/z (C18:0 and C20:3), respectively. This example demonstrates the reliability of discrimination between phosphorylation and sulfation on structurally very similar aliphatic backbones using the negative ion mode MALDI-TOF/TOF method developed in this contribution. This is not possible using more commonly used ion-trap based instruments, due to the inherent instability of the $[\cdot SO3]^-$ radical ion. This novel finding bodes well for the routine distinction between Ser/Thr sulfation and phosphorylation, for which there are currently no reliable proteomics methods.

1. Metelmann, W., Müthing, J., Peter-Katalinic, J. (2000) Rapid Commun. Mass Spectrom. **14**, 543-550.

Supplementary Material

Figure S1

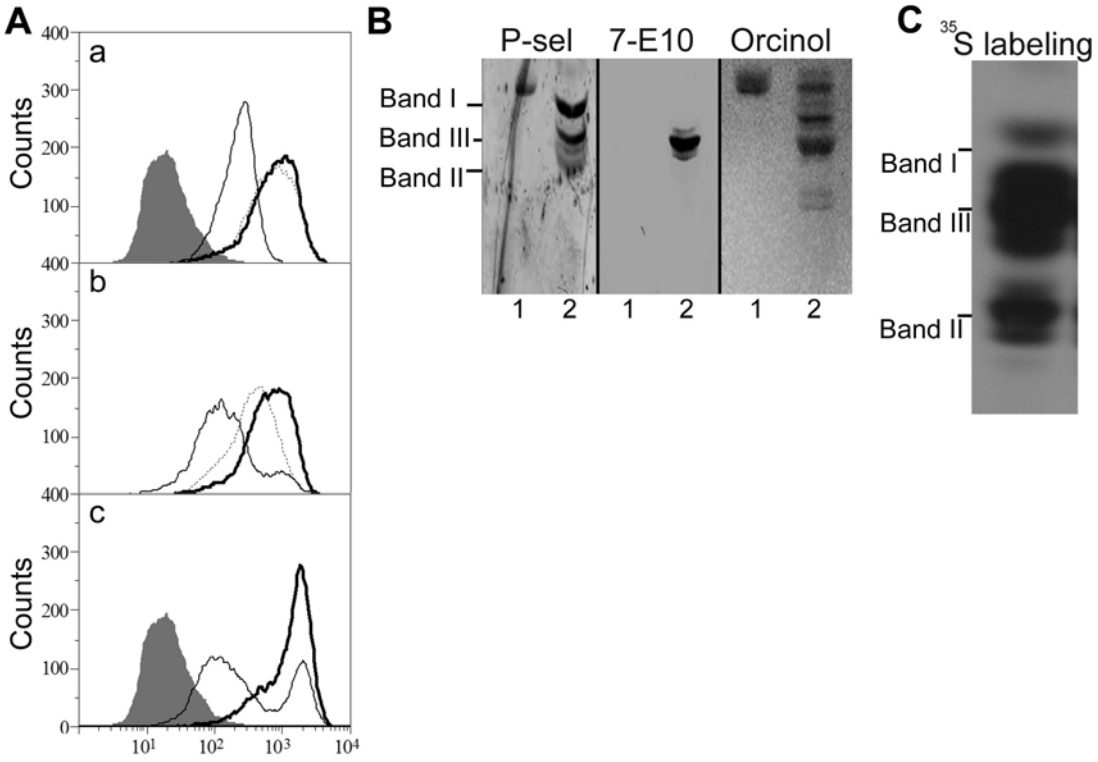


Figure legends to Panel 1A: Bold solid lines represents P-selectin stained HepG2 cells.

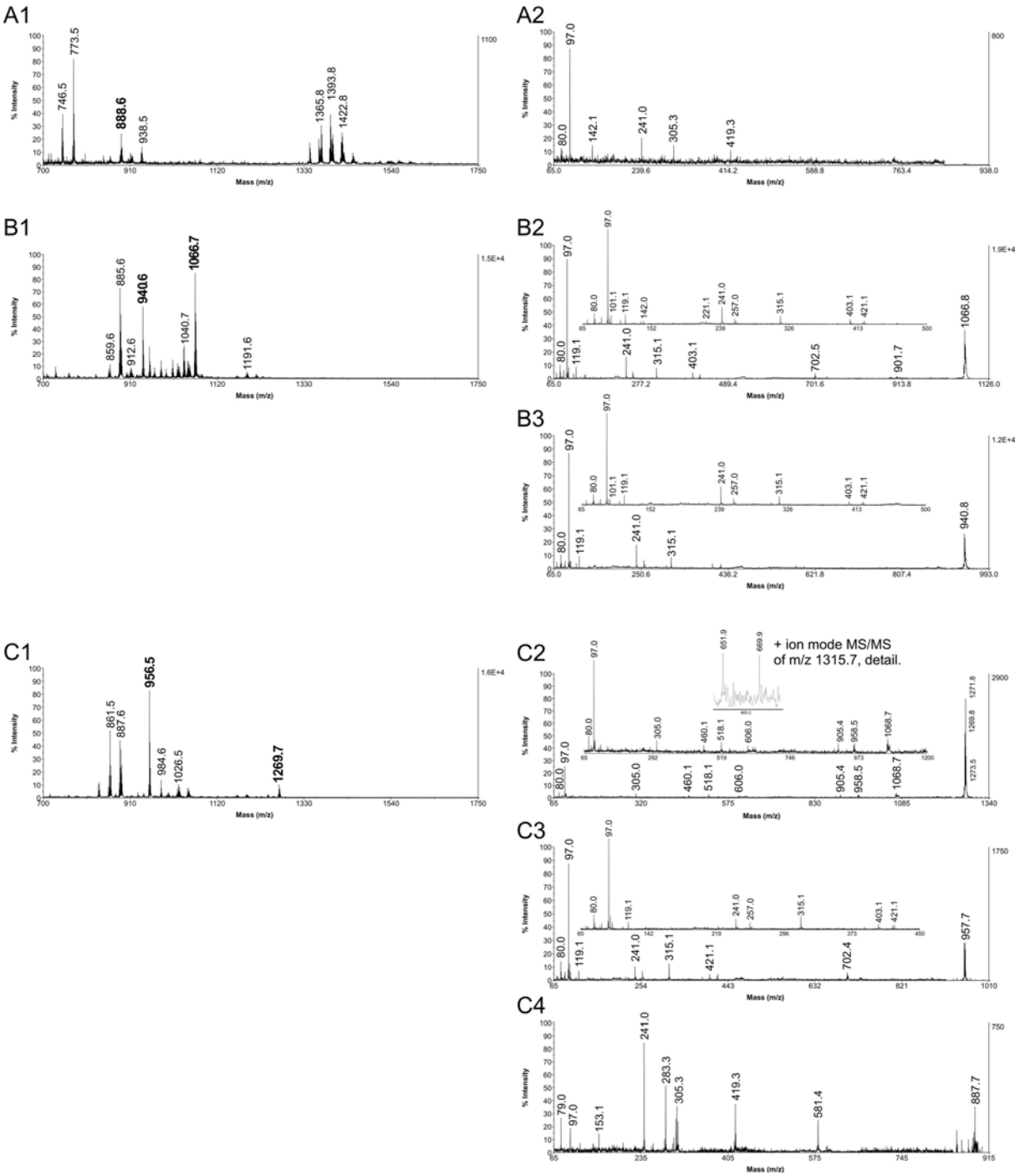
a) thin line - P-selectin staining in the presence of 30mM EDTA
dashed line - OSGP-ase treated cells stained with P-selectin

b) thin line - arylsulfatase treated cells stained with P-selectin
dashed line - heparinase-chondroitinase treated cell stained with P-selectin

c) thin line - P-selectin staining of HepG2 cells treated with Na-chlorate

Supplementary material

Figure S2



Silencing of Cerebroside Sulfotransferase (CST) of MC-38 tumor cells results in a reduction of the metastasis

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University of Zürich, 8057 Zürich, Switzerland

Abstract

The alteration of carcinoma cell surface glycosylation is a common feature and has been correlated with poor prognosis in colorectal carcinoma. Here we show that the expression of sulfatides on mouse colon carcinoma cells (MC-38) correlates with metastasis. SM4 sulfatide was shown to be recognized by P-selectin. *In vitro* data suggested that the recognition of SM4 participates in the platelet-tumor cell aggregation. Cerebroside sulfotransferase (CST) is the enzyme responsible of the synthesis of sulfatides. The inhibition of the synthesis of sulfatides, by siRNA of CST, reduced the ability of tumor cells to adhere to the glycocalix of endothelial cells. Decrease in the sulfatide expression was also associated with diminished platelet-tumor cell aggregation *in vivo* which resulted in attenuation of metastasis. All these results suggest a biological role of carcinoma sulfatides in selectin mediated metastasis.

Introduction

Tumor cell malignancy has been associated with alterations of cell surface glycosylation and correlated with poor prognosis due to high rate of metastases (Kannagi 1997). Besides, carcinogenesis has been associated with expression of glycolipids (Hakomori 1986; Hakomori 1998).

Sulfoglycolipids or sulfatides form part of a class of acidic glycolipids containing sulfate esters on their oligosaccharide chains. They are abundant in myelin, spermatozoa, kidney and small intestine. They have been implicated in a variety of physiological functions through their interactions with extracellular matrix proteins, cellular adhesive receptors, blood coagulation systems, cation transport systems and microorganisms (Ishizuka 1997). Enhanced sulfatide expression was detected in some carcinomas like renal cancer, gastric cancer, colorectal carcinoma (Hiraiwa, Fukuda et al. 1990; Kobayashi, Honke et al. 1994; Morichika, Hamanaka et al. 1996). Transfer of the sulfate group to the glycolipid moiety is mediated by cerebroside sulfotransferase (CST), thus producing sulfatide (Honke, Tsuda et al. 1997; Hirahara, Tsuda et al. 2000). Activity of CST has been associated with cancer in human renal carcinoma cells (Tsuda, Egashira et al. 2000). However the role of sulfatide in cancer malignancy has not yet been defined.

Selectins are vascular adhesion molecules that recognize specific carbohydrate structures. There are three types of selectins: E, L and P-selectin which mediate leukocyte adhesion and transmigration to endothelium, inflammation, thrombosis, autoimmunity and cancer (Varki 1994; Kansas 1996). Similarly, the presence of selectin ligands on tumor cells facilitates their adhesion to endothelium therefore facilitating metastastasis (Kim, Borsig et al. 1999; Kannagi, Izawa et al. 2004). In addition, formation of tumor cell emboli has been shown to be mediated by selectins. (Kim, Borsig et al. 1998) These authors showed that reduction of platelet-tumor cell aggregates was observed in P-selectin deficient mice and this reduction was associated a decrease of metastasis. Similarly the inhibition of P-selectin by heparin resulted in attenuation of metastasis (Borsig, Wong et al. 2001). In absence of L-selectin attenuation of metastasis was also observed which implicates direct involvement of leukocytes in this process (Borsig, Wong et al. 2002).

P and L-selectin are able to efficiently bind sulfatides (Aruffo, Kolanus et al. 1991; Suzuki, Toda et al. 1993; Bajorath, Hollenbaugh et al. 1994; Ishizuka 1997).

Previous data showed that a sulfo-galactosyl ceramide (SM4), expressed on mouse colon adenocarcinoma cells MC-38, is recognized by P-selectin (Garcia, Callewaert et al. 2006). Removal of sulfation on these cells attenuated metastasis. In this work we provide evidence to show that SM4 is a main ligand for P-selectin. Silencing of CST resulted in a decrease in the platelet-tumor cell aggregation and, consequently, in the attenuation of metastasis.

Materials and Methods

Cell lines and reagents

Mouse colon carcinoma cells line MC-38 and MC-38GFP, a population of cells stably expressing GFP (Borsig 2002) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). All reagents unless otherwise stated were from Sigma (St Louis, MO).

Flow Cytometry Analyses of MC-38 cells

Cells were grown to 90% confluency and detached with PBS containing 2 mM EDTA for 5 min at 37°C and washed three times with cold Hank's Balanced Salt Solution (HBSS). Cells were blocked with 1% BSA in HBSS prior to incubation with mouse P-selectin chimera, containing the Fc region of human IgGs (Borsig 2002). P-selectin chimera containing biotinylated goat anti human IgG antibody (Ab) (1:200) was added to tumor cells after being incubated for 1 h. Streptavidin PE-Cy5 (Becton Dickinson, Mountain View, CA) was added to the mixture after 1 h at 4ºC and further incubated for additional 20 min. Stained cells were analyzed by flow cytometry.

Modification of SM4 expression by CST siRNA

CST short hairpin RNAs (shRNAs) were prepared using the siRNA design tool from MWG (Ebersberg, Germany) and extended with restriction sites to enable the cloning in pSuper.retro.puro vector (Oligoengine, Seattle, WA). Two different oligo sequences were used to silence the mouse CST gene; one (CST-297) targeting the nucleotide position of 297, 5'–GATCCCCGAAGCACGAGCTCAAGTTCTTC AAGAGAGAACTTGAGCTCGTGCTTCTTTTTC - 3', and the other (CST-793) one targeting the nucleotide position 753, 5'-GATCCCCGGAGTACTTCGACGAATC CTTCAAGAGAGGATTCGTCGAAGTACTCCTTTTTC -3'. As a scramble siRNA,

a GFP siRNA (Qiagen, Hilden, Germany) sequence was used. MC-38 cells were transiently transfected with shRNA using electroporation with a Nucleofector device from AMAXA biosystems (Cologne, Germany) according to the protocol of the manufacture. Briefly, 3 x 10^6 cells were resuspended in 100 µl of Nucleofector solution together with 2.5 µg of each CST-297 and CST-793 cloned into pSuper.retro.puro vector. The cells were then placed in an electroporation cuvette. Immediately after electroporation, pre-warmed dulbecco's Modified Eagle's Medium **(**DMEM**)** 10% FCS medium was added to the cuvette and the cells were transferred into a culture plate containing pre-warmed medium. The cells were re-transfected 24 h after, following the same procedure. At different time points after transfection, cells were detached with EDTA and stained with P-selectin chimera as described above and analyzed by flow cytometry.

Lung endothelial cells isolation

Endothelial cell isolation was performed as described (Wang, Fuster et al. 2005) and optimized by MA Boucabeille (manuscript in preparation). Briefly, anesthetized mice were injected with PBS/30U Heparin intravenously and cardiac perfusion was performed with PBS (1 x 10 ml). A solution of 0.1% collagenase A was injected through the trachea. Lungs were cut into small pieces and placed in a solution of 0.1% collagenase A in PBS and incubated while shaking at 37ºC. Released cells were recovered by low speed centrifugation; the resuspended pellet was filtrated through a 100 µm sterile mesh. A mAb anti CD31-FITC was added to the filtrated solution containing endothelial cells in suspension and allowed to incubate for 30 min. After washing, the mix was incubated with magnetic beads coated with Ab anti-FITC isotype for 15 min. The sample was poured over a magnetic column. After three times washing with PBS, magnetic field was removed and eluting cells were put onto culture with EC medium, (DMEM low glucose; HEPES 25 mM; non essential amino acids- 1x, penicillin streptomycin 1x; Heparin 100 µg/ml, 20% FCS, ECGS - endothelial cell growth supplement 100 µg/ml)

Assay for attachment and transmigration of tumor cells on endothelial cells

Primary cultured lung endothelial cells (10^5 cells) were seeded on the 8-well cell culture inserts (apical chamber, Falcon) with a pore size of 8 µm and maintained in

CS-C medium overnight. After replacing the medium with DMEM with 10% FCS, 25 x 10^4 MC38-GFP cells or transfected with siRNA-CST MC38-GFP cells were added to the apical chambers. After 3 h at 37ºC, all apical chambers were washed gently with PBS twice in order to remove the non-attaching tumor cells to the endothelial layer. The apical chambers were fixed with 3% paraformaldehyde (PFA) in PBS for 15 min on ice and afterwards stained with 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) for 20 min before cutting the inserts and mounting them on slides for counting the number of MC38-GFP cells attached on endothelial cells by a fluorescence microscope. Additional apical chambers were further incubated to determine the trans-endothelial migration of MC38-GFP cells. For this experiment, the apical chamber was incubated with DMEM without FCS while the lower chamber contained DMEM with 10% FCS. After incubation for 16 h, non-migrating cells were removed by scraping the apical side of the apical chambers with a cotton swab. Then, the apical chambers were fixed and stained with DAPI and subjected to the examination of transmigration of MC38-GFP cells by a fluorescence microscope.

In Vivo interactions of siRNA-CST transfected tumor cells with platelets

The procedure was done as previously described (Borsig, Wong et al. 2001) (Garcia, Callewaert et al. 2006). Briefly, lungs were obtained for analysis 30 min. after tail vein injection of MC38-GFP cells, (mock-transfected or with siRNA-CST). Lungs sections were stained with rat anti mouse CD41 Ab (BectonDickinson) followed by Goat anti Rat Ab- Alexa568 (Invitrogen, Carlsbad, CA). Platelet-tumor cell association was quantified by counting 40 view fields under fluorescence microscope.

Experimental metastasis assays

The procedure was carried as previously reported (Borsig, Wong et al. 2002; Garcia, Callewaert et al. 2006). Briefly, mice were injected via the tail vein with 3 x 10^5 of MC-38-GFP transfected cells with CST-siRNA or with scramble siRNA. After two weeks lungs were dissected, the number of metastatic foci was counted and a picture was taken.

Results

CST silencing in MC-38 cells reduces P-selectin binding and attenuates metastasis.

Previously, we have shown that MC-38 cells possess the sulfatide SM4 on their surface and that it is recognized by P-selectins (Garcia, Callewaert et al. 2006). Cerebroside sulfotransferase (CST) is the key enzyme in the synthesis of sulfatides (Hirahara, Tsuda et al. 2000). Removal of sulfate with arylsulfatase treatment from MC-38 cell surface was correlated with an attenuation of metastasis (Garcia, Callewaert et al. 2006). Since desulfation is a broad treatment that does not only target sulfatides but also other sulfate groups on the cell surface, we aimed at specifically silencing CST by using siRNA. First we observed that *in vitro* down-regulation of SM4 expression on MC38 cells resulted in reduced P-selectin binding, confirming that SM4 is the main P-selectin ligand on these cells (Fig. 1). This decrease on P-selectin binding was in accordance with the decreased obtained after desulfation of MC-38 cells as described previously (Garcia, Callewaert et al. 2006).

Second, we concluded with in vivo studies for the evaluation of the role of SM4 in metastasis. Therefore mice were injected into the tail vein with MC-38 cells expressing CST-siRNA or scramble siRNA. Two weeks after the injection, mice were euthanized, lungs were extracted (Figure 2 A) and the number of foci was counted (Figure 2B). The number of metastatic foci in the lungs was significantly lower as a result of the inhibition of the expression of SM4 in tumor cells (Figure 2A, B), suggesting that the presence of SM4 promotes metastatic progression, probably mediated by selectin binding. Since long the role of platelet - tumor cell aggregation has been correlated with facilitation of metastasis playing an important role protecting tumor cell from the innate immune response (Karpatkin and Pearlstein 1981; Nieswandt, Hafner et al. 1999). To determine if the platelet-tumor cell aggregation was altered by the lack of SM4, MC38-GFP expressing scramble siRNA or CST-siRNA were injected into the tail vein of mice. Mice were euthanized and lungs were extracted 30 minutes after injection. Staining of lung sections by platelet specific Ab (CD41) showed a reduction in the amount of tumor cells that were aggregated with platelets; which correlated with the lower expression of P-selectin ligand suggesting that sulfatides mediates P-selectin platelet aggregation (Figure 3). Behavior *in vitro* of MC-38 cells, such as division time and adhesion on plastic, did not change as a consequence of the lack of SM4, (data not shown).

Our results are in accordance with previous observations where the impairment of platelet-tumor cell aggregation mediated by P-selectin resulted in reduction of the metastatic progression (Borsig, Wong et al. 2001).

Adhesion of MC-38 cells on endothelial cells is altered by the decrease of SM4 expression

We wanted to study *in vitro* whether the adherence on endothelial cells of MC-38 cells lacking SM4 was altered. MC-38 cells were incubated on a monolayer of mouse lung endothelial cells for 3 h. The adherent cells were fixed and counted by fluorescence microscopy. MC-38 cells lacking SM4 on their surface adhered significantly less on endothelial cells than control MC-38 cells (Figure 4), while there was no difference in adhesion of MC-38 cells to plastic (data not shown). These results suggest that the difference in adhesion to endothelial cells was due to specific interactions mediated by SM4 expressed on MC-38 cells.

Discussion

Glycolipid expression appears particularly affected in tumor cells which acquired metastatic phenotype; these molecules play an essential role in the interactions between tumor cells and the host tissues during the metastatic cascade (Ruggieri, Mugnai et al. 1999).

Sulfatides are implicated in a variety of physiological functions through their interactions with extracellular matrix proteins, cellular adhesive receptors; they have also been shown to be increased in number of human carcinomas (Ishizuka 1997). It has been suggested that sulfatides may participate in platelet aggregation and leukocyte activation (Merten and Thiagarajan 2001; Shimazawa, Kondo et al. 2005). SM4 is the most abundant sulfatide in mammals (Tadano-Aritomi and Ishizuka 2003). The expression of sulfatides is mainly controlled at the transcriptional level of the CST gene. Over-expression of this gene in a human hepatoma cell line resulted in increased intrahepatic metastasis and higher adhesive ability to extracellular matrix proteins (Zhong Wu, Honke et al. 2004). These data suggested the involvement of sulfatide in cellular adhesion. However, the role of sulfatides during metastasis is not well understood. Previously, we could show that a mouse colon carcinoma cell line MC-38 expressed SM4 on its surface and that this SM4 could be recognized by P-

selectin (Garcia, Callewaert et al. 2006). In our present work CST gene was silenced to inhibit the expression of SM4 on MC-38 cells (Figure 1). Down-regulation of the expression of sulfatide was correlated with a decrease in the metastatic progression of MC-38 cells (Figure 2A, B). Previous studies showed the role of P-selectin as a mediator in the metastatic process through recognition of a ligand expressed on the tumor cell surface (Borsig, Wong et al. 2002; Garcia, Callewaert et al. 2006). In this work a direct link could be established between the expression of SM4 on MC-38 and its role facilitating metastasis through P-selectin recognition. As a consequence of the lack of SM4 the aggregation of platelets with tumor cells was altered (Figure 3). These data indicate that sulfatides expressed on tumor cells may act as a main ligand for platelet P-selectin mediating the platelet-tumor cell emboli formation. The formation of these aggregates have been shown to participate in the metastatic process (Honn, Tang et al. 1992; Kim, Borsig et al. 1998), and the blockage of this process resulted in attenuation of metastasis (Varki and Varki 2001). The inhibition of the expression of SM4 on MC-38 cells also altered the adhesion capability on endothelial cells *in vitro* (Figure 4). Still remains to be elucidated what the mechanisms participating in this process are. There are also other questions that remain to be answered: how often are sulfatides over-expressed in other carcinomas? Are sulfatides on tumor cells able to activate platelets? Other studies will be needed to further elucidate the role of sulfatides during cancer progression.

Although many questions still remain unanswered, we could directly link sulfatide expression with metastatic capacity. These results suggest the possibilities to use sulfatide as a marker for malignancy as well as a possible target to develop future therapies, provided that analogue interactions could be delineated in humans.

Figure Legends

Figure 1 *P-selectin binding to MC-38 cells following down-regulation of CST.* MC-38 cells were labeled with mouse P-selectin chimera and analyzed by flow cytometry. Filled area represents control stained with the Streptavidin PE-Cy5 only. The thick line represents P-selectin staining of non-transfected cells. Thin line represents P-selectin staining of cells transfected with siRNA for CST, as described in the material and methods section.

Figure 2 *Down-regulation of SM4 expression attenuates metastasis.* Mice were injected intravenously with 3-4 x 10^5 MC-38GFP cells either transfected with scramble siRNA or with siRNA for CST. Mice were euthanized two weeks following injection, lungs were dissected and photographed (**A**). The number of animals studied was 5 in each group. The extent of metastasis was measured by counting the number of tumor foci in each lung (**B**). Statistical significance was determined by Student's t-test.

Figure 3 *Platelet-tumor cell association is decreased by the lack of SM4.* Platelet interactions with injected cells were evaluated in lungs from mice terminated 30 min after injection. Platelet-tumor cell interactions were quantified by counting tumor cells in 40 view fields by fluorescence microscopy.

Figure 4 *The adhesion of MC-38 cells to endothelial cells in vitro is altered by down-regulation of SM4*. Lung endothelial cells were grown to confluence on a 8.0 mm pore size membrane before adding 25.000 MC38-GFP tumor cells either non-transfected or transfected with of siRNA for CST, as described in the material and methods section. After three hours of co-incubation non attached cells were washed gently and the adherent cells were fixed and stained with DAPI. Attached cells were counted at the microscope 10 view fields by 63x magnification.

Figure 1

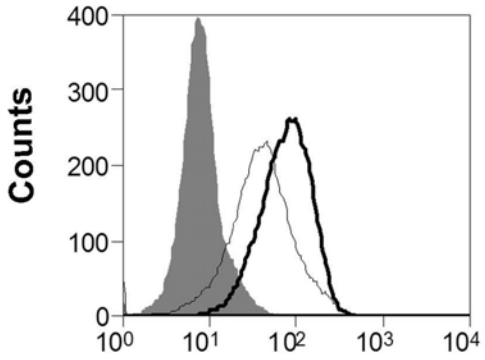


Figure 2

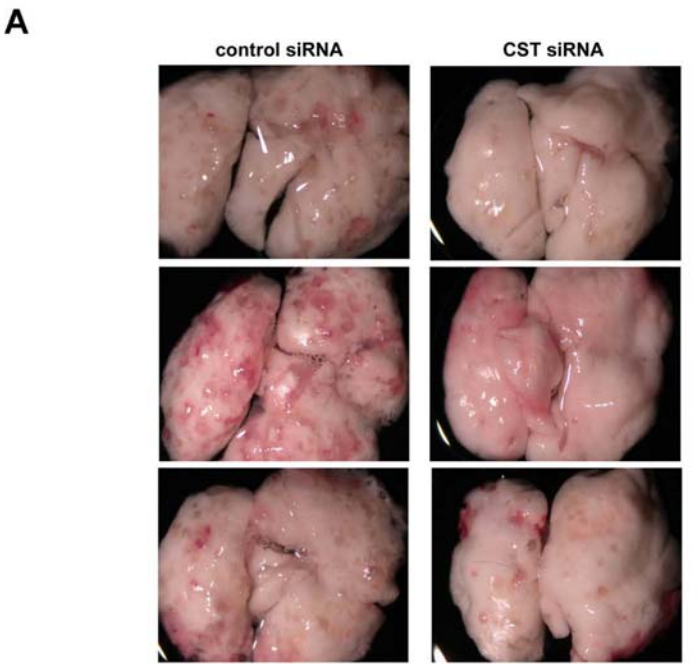

A
control siRNA
CST siRNA
B
Metastatic foci
150
100
50
0
p <0.01
contr
CST

Figure 3

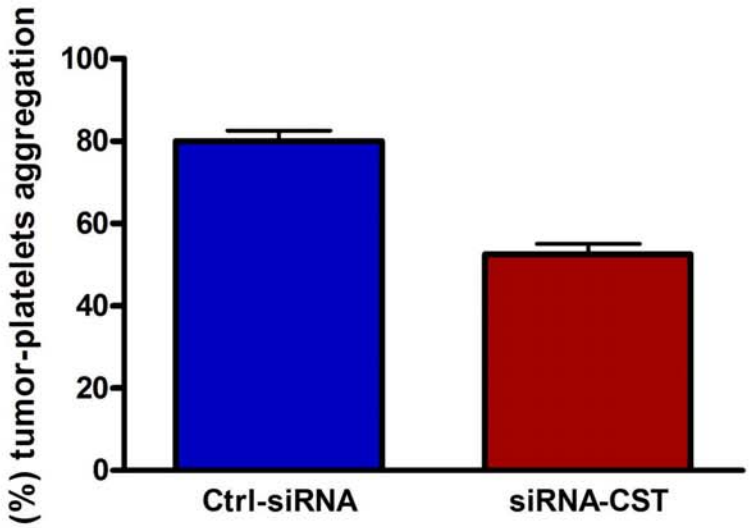
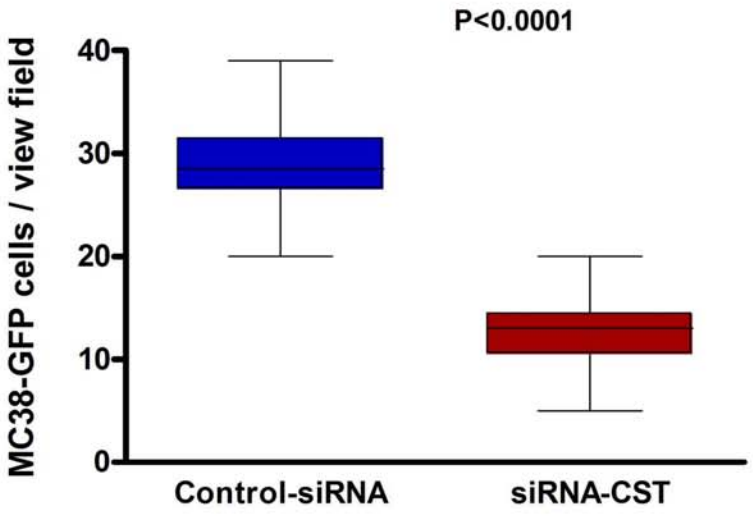

(%) tumor-platelets aggregation
100
80
60
40
20
0
Ctrl-siRNA
siRNA-CST

Figure 4


P<0.0001
MC38-GFP cells / view field
40
30
20
10
0
Control-siRNA
siRNA-CST

Section III

Discussion

Discussion

Malignacy, the process by which cancer cells invade and metastasize, require that cancer cells undergo a battery of multiple changes. Besides the many changes affecting cell cycle, metabolism and polarity, a common change in carcinoma is the alteration of cell surface glycosylation. The high expression of sialylated and fucosylated glycans on mucins has been associated with tumor progression and enhanced metastasis (Dennis, Granovsky et al. 1999; Hollingsworth and Swanson 2004; Dube and Bertozzi 2005). Not only alterations in mucins but also in glycosaminoglycans and glycolipids have been associated with changes on the cell surface glycosylation of tumor cells. Glycolipids are molecules involved in cell adhesion. Specific glycolipid changes on metastatic cells have been shown to play a crucial role for the invasive nature, probably through interactions of the tumor cell with their microenvironment (Hakomori 1986; Ruggieri, Mugnai et al. 1999).

In this work, we showed that a special class of glycolipids, the sulfatides, is involved in the process of metastasis. Sulfatides have often been associated with cancer and also correlated with malignancy. SM4 is the most abundant sulfatide found in mammals, particularly, in brain and in the gastric mucosa (Natomi, Saitoh et al. 1993; Tadano-Aritomi and Ishizuka 2003). Sulfatides were shown to interact with extracellular matrix proteins and cellular adhesive receptors (Ishizuka 1997; Tsuda, Egashira et al. 2000). Recent studies have assigned a new role for sulfatides in platelet aggregation and leukocyte activation (Merten and Thiagarajan 2001; Shimazawa, Kondo et al. 2005). Several human cancer tissues, including lung (Miyake, Taki et al. 1992), colon (Siddiqui, Whitehead et al. 1978; Osawa, Sugano et al. 1997), kidney (Sakakibara, Gasa et al. 1989), liver (Hiraiwa, Fukuda et al. 1990) and ovary (Kiguchi, Takamatsu et al. 1992) presented high expression of sulfatides. Although enhanced expression of sulfatides was correlated with poor prognosis (Morichika, Hamanaka et al. 1996; Makhlouf, Fathalla et al. 2004) their biological role in cancer is not yet fully understood.

In this work we have studied and characterized the role of SM4 sulfatide as key modulator of metastasis. SM4 was identified to be the main P-selectin ligand on colon carcinoma cells (MC-38) as determined by MALDI-TOF-TOF analysis of purified glycolipids. Sulfatides were already shown to be recognized by selectins

(Aruffo, Kolanus et al. 1991; Nelson, Cecconi et al. 1993; Suzuki, Toda et al. 1993; Kawashima, Hirose et al. 2000). Selectins are vascular adhesion molecules involved in leukocyte trafficking, inflammation, thrombosis, autoimmunity, and cancer (Kansas 1996). The binding of selectins to sulfatides was described to participate in different cellular processes such as mediation of the infiltration of monocytes in kidney (Ogawa, Shikata et al. 2004), activation and aggregation of platelets (Merten and Thiagarajan 2001; Merten, Beythien et al. 2005). P-selectin has been shown to bind only SM4 sulfatide (Aruffo, Kolanus et al. 1991), while L-selectin was shown not only to bind SM4, but also sulfated lactosylceramide SM3, and sulfated tri- and tetra-glycoceramides SM2 and SB1a, respectively (Suzuki, Toda et al. 1993). High expression of the different sulfatide structures have been found in several cancers (Kobayashi, Honke et al. 1994; Ishizuka 1997; Zhong Wu, Honke et al. 2004). We could prove by using purified glycolipids of a human hepatoma cell line (HepG2) that P-selectin can also recognize SM3 and SM2a *in vitro*. HepG2 cells were used as a model of sulfatide expressing cells for our *in vitro* assays since they presented a similar P-selectin binding pattern as MC-38 cells. These results show that P-selectin can recognize sulfatides other than SM4, indicating that broader sulfatides could be participating in selectin-mediated actions in metastasis.

Selectin binding to ligands expressed on tumor cells is known to actively participate in the metastatic process. Heparin is a known inhibitor of selectins which can bind transiently, therefore blocking the binding of selectins to other relevant ligands (Nelson, Cecconi et al. 1993; Koenig, Norgard-Sumnicht et al. 1998; Borsig, Wong et al. 2002; Kragh and Loechel 2005). We previously described in our model that the absence or blockage of P-selectin resulted in attenuation of metastasis (Kim, Borsig et al. 1998; Borsig, Wong et al. 2001; Ludwig, Boehme et al. 2004). Desulfation treatment of MC-38 cells decreased P-selectin binding *in vitro* and strongly attenuated metastasis *in vivo*. The reduction of metastasis was correlated with a decrease of platelet-tumor cell aggregation. This data is in line with previous studies where the inhibition of P-selectin by heparin or removal of the selectin ligands resulted in an impairment of the interactions between tumor cells and platelets and/or endothelium and consequently in attenuation of metastasis, (Borsig, Wong et al. 2001; Borsig, Wong et al. 2002; Fuster, Brown et al. 2003; Ludwig, Boehme et al. 2004). Our results suggested that SM4 is the ligand recognized by P-selectin on MC-38 cells and this binding facilitates metastasis through adhesion to platelets.

The presence of sulfatides on cancer cells was correlated with the high expression of ceramide sulfotransferase gene - CST (Honke, Tsuda et al. 1998) and associated with increased metastatic potential of human hepatocellular carcinoma cells (Zhong Wu, Honke et al. 2004). The recent finding that sulfatides could lead to platelet activation and their aggregation with leukocytes suggesting that this mechanism plays an important role in hemostasis and thrombosis (Merten and Thiagarajan 2001; Merten, Beythien et al. 2005). Silencing of CST gene on MC-38 cells resulted in a decreased P-selectin binding and to attenuated metastasis. This attenuation was also correlated with a decrease in platelet-tumor cell aggregation. These results are in agreement with the effect obtained by desulfation treatment of MC-38 cells. In this context, our finding that murine colon carcinoma cells carrying sulfatides can effectively induce P-selectin-mediated platelet aggregation raises the possibility for the involvement of these interactions during hematogenous metastasis, since this process is reduced in the absence of sulfatides.

Sulfatides can be bound by extracellular matrix proteins. That is the case for human renal-cell carcinoma and hepatocellular carcinoma cells which were shown to interact with laminin through sulfatides (Kobayashi, Honke et al. 1994; Zhong Wu, Honke et al. 2004). Renal cancer cells incubated in an excess of SM4 showed increased migration on laminin and enhanced lung colonization (Kobayashi, Honke et al. 1994). The presence of SM3 sulfatide was linked to the up-regulation of CST gene expression, which also mediated cell adhesion to vitronectin and $\alpha V\beta 3$ integrins (Honke, Tsuda et al. 1998; Zhong Wu, Honke et al. 2004). Recently, sulfatides were found to be potential native ligands for Galectin-4, a galactose-binding lectin (Ideo, Seko et al. 2005). The role of sulfatides on cancer cells in adhesion has been shown in several works (Kabayama, Ito et al. 2001; Zhong Wu, Honke et al. 2004). Expression of sulfatides by human hepatocellular carcinoma enhanced metastasis by cell adhesion to vitronectin (Kabayama, Ito et al. 2001; Zhong Wu, Honke et al. 2004). Other work showed that the expression of sulfatide in Lewis lung carcinoma resulted in reduction of adhesion and in a decrease of metastasis (Kabayama, Ito et al. 2001; Zhong Wu, Honke et al. 2004). Altogether these data suggest an important role of sulfatides in adhesion of tumor cells to extracellular matrix proteins and the discrepancy between previous results on adhesion and metastasis may be due to differences of the physiology of these types of cancer cells. Our results show greater attenuation of

metastasis by desulfation or silencing CST of MC-38 cells than the attenuation obtained in P-selectin deficient mice. These observations suggest that there are other mechanisms in which sulfatides may contribute to facilitating metastasis. This hypothesis was supported by i*n vitro* evidence showing a decreased adhesion to a monolayer of lung endothelial cells of MC-38 cells lacking sulfatide. The nature of these interactions is the object of a current study. More experiments are in progress in order to investigate the specificity of these results and their link with sulfatide expression.

The negatively charged phospholipid cardiolipin (CL) was also found to be isolated from MC-38 cells. CL is normally localized in the inner mitochondrial membrane (Schlame, Rua et al. 2000). However, CL has also been detected on the cell surface of apoptotic cells after redistribution from mitochondria (Sorice, Circella et al. 2004). We have shown that significant amounts of CL were found in MC-38 cell extracts, while CL usually comprises a minor component of the total lipid extracts in other cell lines such as HepG2 cells or human cancer carcinoma (LS-180), in which virtually no CL could be detected. Previous studies have reported L-selectin binding of CL in calcium-independent manner (Malhotra, Taylor et al. 1996). P-selectin could bind CL isolated from MC-38 cells. The major question that remains to be answered is whether CL is expressed on the plasma membrane and therefore could be a biological ligand for P-selectin. The lack of specific antibody did not allow us to continue further in this direction. It is an interesting observation that we were able to visualize a considerable high amount of CL when compared with other cell lines; however the biological relevance of this finding remains unclear. Even though there is a possibility that CL could be recognized by P-selectin, all our data points SM4 as the main P-selectin ligand on MC-38 cells. Thus, the relevance of sulfatides is validated as active players in the mediation of metastasis

This work shows the biological function of sulfatides expressed on cancer cells, which can mediate the metastatic process by P-selectin recognition. The binding of sulfatides with P-selectin facilitates the formation of the platelet-tumor cell aggregates, known to be an important step in metastasis facilitating interactions with leukocytes and endothelial cells. Platelet aggregation around the tumor cell provides a protective coating that can prevent a native immune response. It was shown that natural killer cell-mediated lyses of tumor cells is blocked by platelets *in vitro* and in mouse tumor models (Nieswandt, Hafner et al. 1999; Trikha and Nakada 2002;

Laubli, Stevenson et al. 2006). How sulfatides participate in the adhesion capability of MC-38 cells to endothelium remains to be clarified.

These results establish a new player in the complex process of selectin-mediated metastasis. Different mechanisms could still be participating in the role of sulfatides in cancer. Further studies are required to understand the molecular mechanisms behind the effect of sulfatides in metastasis. Studies of membrane distribution of sulfatides are needed to determine if they are part of microdomains -lipid rafts- (Sandhoff, Grieshaber et al. 2005) and whether they could participate in signal transduction.

Even though there are still some open questions our results open a new door for the understanding the role of sulfatides in cancer. The fact that has been observed high expression of sulfatides in human cancer such as lung, renal, colon, (Sakakibara, Gasa et al. 1989; Morichika, Hamanaka et al. 1996; Kobayashi, Honke et al. 1999; Zhong Wu, Honke et al. 2004), also raises the possibility for using the expression of sulfatides as a marker for malignancy.

Section IV

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